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13. ABSTRACT *(Maximum 200 Words)*

Bone represents a common site for metastasis of prostate cancer cells, where the invading cells find themselves in an environment rich in factors which promote cancer growth and progression. Genetic changes occur during disease progression in bone which include both gene mutation and changes in the pattern of gene expression. These genetic alterations provide targets for new "molecular drugs" for metastatic prostate cancer. This project will investigate the role of an extracellular matrix protein, osteopontin, which is expressed by metastatic prostate cancer cells but not by normal prostate in the ability of prostate cancer cells to form metastases in bone. The functional properties of osteopontin, including interactions with the $\alpha_v\beta_3$ integrin and CD44 receptor, indicate that osteopontin may play an important role in tumor cell attachment, invasion and growth in the bone environment. A series of ribozymes which specifically cleave OPN mRNA sequences will be developed that will inhibit expression of the osteopontin gene. A series of experiments will be performed in stably transfected prostate cancer cell lines to determine if ribozyme-mediated destruction of the osteopontin gene product modulates cell phenotype. In particular we will examine effects on adhesive properties, growth in soft agar, cell growth rates, chemotaxis, and invasion. These studies will be designed to translate into improved therapies aimed at inhibiting the development of prostate cancer at secondary bony sites. They will also allow development of a prototype for molecular constructs that could be targeted for treatment of other types of cancer that can metastasize to the bones.

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Table of Contents

Introduction: Bone represents a common site for metastasis of prostate cancer cells, where the invading cells find themselves in an environment rich in factors that promote cancer growth and progression. Genetic changes occur during disease progression in bone which include both gene mutation and changes in the pattern of gene expression. These genetic alterations provide targets for new "molecular drugs" for metastatic prostate cancer. This project investigated the role of the bone microenvironment, including an extracellular matrix protein, osteopontin, that is expressed by metastatic prostate cancer cells but not by normal prostate, in the ability of prostate cancer cells to form metastases in bone. The functional properties of osteopontin, including interactions with the $\alpha_v\beta_3$ integrin and CD44 receptor, indicated that osteopontin might play an important role in tumor cell attachment, invasion and growth in the bone environment. Two active ribozymes that specifically cleave OPN mRNA sequences were developed. During the course of the work, we found that growth of prostate cancer cell lines on collagen I (bone collagen type) stabilized the osteoblast transformation of prostate cancer cells including osteopontin expression and stimulated proliferation. We examined effects of osteopontin and collagen type I on the adhesive properties, survival, cell growth rates, chemotaxis, and intracellular signals. We believe the results that we obtained will translate into improved therapies aimed at inhibiting the development of prostate cancer at secondary bony sites. They will also support the development of a prototype for molecular constructs that could be targeted for treatment of other types of cancer that can metastasize to the bones. Unfortunately, our application for continued support of this project was not approved, so this represents the final report.

Body: The research accomplishments are detailed below. To the best of our ability, we follow the organization set forth in the original statement of work. Some studies were postponed because of negative experimental findings or unanticipated technical difficulties. Other exciting new observations were made and incorporated into the experimental plan. Those results that were published are included in the appendix. The specific information requested is provided by task and milestone. Note that all data are consistent with the original hypothesis.

Task 1. Design and clone ribozymes with inducible promoters.

Comment: We designed and cloned two active ribozymes. A third would not clone out for reasons that we have not determined. We have shifted from a strategy to employ an inducible promoter to one that employs a constitutively active promoter because we found the inducible promoter to be "leaky". The ribozymes that we have made are active *in vitro*, and cleave the osteopontin transcript *in vitro*. The sequences of the ribozymes are as predicted, and the constructs appear to be stable. We feel that the two ribozymes we have made and cloned are equally good candidates for later studies. **This completed task 1.**

Task 2. Transfect OPN ribozymes into prostate cancer cells.

Comment: We successfully cloned both active ribozymes into PC-3 cells and selected stable colonies expressing the ribozyme construct constitutively. The assay employed to detect the ribozyme insert and the plasmid vector was a polymerase chain reaction based assay with specific primers. The cell lines expressing the ribozymes are

maintained in neomycin selection medium and appear to be completely viable. We have optimized conditions for transfection. The aim to demonstrate inducible expression was dropped because of the leakiness of the inducible promoter in the presence of androgen used to maintain some androgen-dependent lines such as LNCaP. We felt that switching to constitutively active constructs would allow us to directly compare androgen-dependent and androgen-independent lines as stated in our original specific aims. **This completed task 2.**

MILESTONE 1: Achieved, proceeded to task 3.

Task 3. Confirm ability of ribozymes to regulate levels of OPN expression.

Comment: We had no problem growing cells in the presence of active ribozyme so long as the cells were maintained in selection medium. We developed an RT-PCR assay to quantitate levels of OPN transcript in various cell lines, and have also used a dot blot assay and Northern blots for quantitation. There have been no problems extracting RNA containing full length transcripts encoding OPN. We also developed assays to quantitate levels of protein expression by Western blotting using polyclonal anti-OPN antibodies made in goat. FACS analysis has been used to demonstrate cell surface expression of the OPN protein. These techniques were used to assess the levels of OPN transcript and protein in various prostate cancer cell lines, and demonstrate increased expression associated with disease progression. These data are presented in manuscript 1 included as part of a previous progress report (Thalmann et al, 1999). We assessed the levels of OPN transcript and protein expression in the ribozyme-transfected and parental PC-3 cells. **Task 3 was completed with the changes noted.**

MILESTONE 2: We partially achieved milestone 2. We delayed studies with ribozyme transfected cell lines in light of our exciting discovery of the profound influence of the collagenous matrix on OPN expression (Kiefer and Farach-Carson, appendix). In our continuation application that was not funded, we had hoped to finish these studies.

Task 4. Begin biological assays to determine the role of OPN in various assays. Compare the behavior of control and ribozyme-expressing cell lines.

Comment: We developed reliable assays to measure the effects of OPN on cell adhesion, proliferation and survival, attachment to matrix, and focus formation in soft agar. Two very interesting and unexpected results emerged from these assays. First, we found that OPN itself stimulates a growth response in prostate cancer cells, particularly those conditioned to grow in bone matrix. As an index of growth signal, we devised an assay to measure the ability of bone matrix proteins to stimulate the development of calcium transients in PC-3 cells. These assays employed the fluorescent indicator fura-2, and clearly showed that OPN stimulated a transient that could be blocked by LM-609, an antibody that blocks the function of the integrin, $\alpha_v\beta_3$. These results were submittted and published, (Lecrone et al., appendix). A second very interesting finding is that the expression of OPN by prostate cancer cell lines is dependent on the matrix on which the cells are cultured. We performed a systematic evaluation of the role of the collagen matrix for growing PC-3 cells and maintaining high OPN (and other bone matrix proteins such as OCN) expression levels. We also developed active ribozymes directed at the

collagen receptor, $\alpha_2\beta_1$. These studies are ongoing and we will seek further funding for this exciting work.

Tasks 5 & 6. Data analysis and report writing.

Comment: Relevant manuscripts are attached, and we have submitted all reports including this final report. In addition, we participated in a collaboration to investigate the effects of another matrix protein, laminin, on prostate cancer cell behavior. This manuscript (Edlund et al, 2001) is also attached.

Key Research Accomplishments:

1) Design and cloning of two active ribozymes directed specifically toward OPN and two active ribozymes toward the α_2 integrin. The latter was not part of the original statement of work but was supported by this grant. We felt the results were sufficiently important that this avenue should be investigated.

2) Transfection and stable expression of OPN ribozymes in PC-3 cells.

3) Development of assays for reliable detection of OPN protein and transcript in prostate cancer cells.

4) Development of bioassays for adhesion, proliferation, differentiation and growth stimulation of various prostate cancer cell lines in culture. Study of OPN effects on these behaviors. We had hoped to continue this and combine it with our new knowledge of collagen matrix effects.

5) Demonstration that OPN biosynthesis correlates with increased metastatic potential.

6) Demonstration that OPN expression is dependent upon the content of the biological matrix upon which the cells are growing.

7) Demonstration that collagen I supports the osteoblastic transformation of prostate cancer cells, stimulates proliferation, and promotes survival. Demonstration of role of key signaling pathways in matrix effects (see Kiefer and Farach-Carson, appendix).

Reportable Outcomes:

Manuscripts.

1. Thalmann et al, 1999 (submitted previously)
2. Lecrone et al (appendix).
3. Edlund et al (appendix).
4. Kiefer and Farach-Carson (appendix).

Abstracts.

The work in Kiefer and Farach-Carson was presented in abstract (poster) form at the annual meeting of the American Society for Bone and Mineral Research in Toronto, 2000.

Patents and Licenses.

Not applicable.

Degrees obtained.

Mr. Jeff Kiefer is a full time graduate student defended his Ph.D. dissertation in April, 2001. He will pursue a postdoctoral experience in Seattle beginning this summer.

Cell lines.

The ribozyme-transfected cell lines will be available to other researchers at the conclusion of these studies. They are not yet completely characterized.

Databases and animal models.

Not applicable.

Funding.

We will seek continued funding for this project through various mechanisms.

Training.

Dr. Wei Li, who was a research associate working in part on this project, left the lab to pursue a residency in pathology at the University of Texas-Houston, Medical School. He will continue his clinical interest in cancer biology in this new clinical role.

Several undergraduates at the University of Delaware including Ms. Jennifer Morrison and Ms. Kate Gamblee-Wallendjack worked on aspects of this project and received summer fellowships from this university to support their work. These individuals presented their work at undergraduate research symposia at the University of Delaware.

Conclusions:

Our long term goal is to develop ribozyme-based strategies to reduce survival, growth and progression of prostate cancer cells that are growing in a bone extracellular matrix environment such as that encountered by metastatic prostate cancer cells growing at bony sites. These molecular strategies should be amenable to combination with other established therapies including surgery and hormone ablation. We believe we have made progress toward this aim, and plan to continue our studies as soon as other funding can be obtained. *We conclude that the matrix has a profound influence on prostate cancer cell*

behavior and maintain that ribozyme disruption of these cell-matrix interactions should be detrimental to cancer progression.

References:

A key paper recently appeared supporting our data and hypothesis.

Nemoto, H., Rittling, S.R., Yoshitake, H., Furuya, K., Amagasa, T., Tsuji, K., Nifuji, A., Denhardt, D.T. and Noda, M. Osteopontin deficiency reduces experimental tumor cell metastasis to bone and soft tissues. J. Bone Min. Res. 16: 652-, 2001.

Appendices:

Lecrone, V., Li, W., Devoll, R.E., Logothetis, C. and Farach-Carson, M.C. Calcium signals in prostate cancer cells: specific activation by bone-matix proteins. Cell Calcium 27:35-42, 2000.

Edlund, M., Miyamoto, T., Sikes, R.A., Ogle, R., Laurie, G.W., Farach-Carson, MC., Otey, C.A., Zhau, H.E. and Chung, L.W..K. Integrin expression and usage by prostate cancer cell lines on laminin substrata. Cel Growth & Differentiation 12: 99-107, 2001.

Kiefer, J.A. and Farach-Carson, M.C. Type I collagen-medicated proliferationof PC3 prostate carcinoma cell line: implications for enhanced growth in the bone microenvironment. Matrix Biology, *submitted (in revision).*

Research

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Calcium signals in prostate cancer cells: specific activation by bone-matrix proteins

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Summary Cancer of the prostate commonly metastasizes to bony sites where cells acquire an aggressive, rapidly proliferating, androgen-independent phenotype. The interaction between bone and prostate, thus, becomes a key factor in disease progression. Fluctuations in intracellular ionized Ca^{2+} $[Ca^{2+}]_i$ are rapid, regulated signal transduction events often associated with cell proliferation. Hence, Ca^{2+} signals provide a convenient measure of early events in cancer cell growth. This study developed single cell fluorescent imaging techniques to visualize Ca^{2+} signals in Fura-2 loaded prostatic cancer cell lines of various metastatic phenotypes. Solubilized bone fractions containing extracellular matrix and associated proteins were tested for the ability to trigger Ca^{2+} signals in prostate cancer cell lines. Fractions representing the complete repertoire of non-collagenous proteins present in mineralized bone were tested. Results demonstrated that two bone fractions termed D3b- and D4a-triggered Ca^{2+} signals in prostate cancer cells derived from bone (PC-3), but not brain (DU-145) metastases of prostate cancer. Lymph-node derived LNCaP cells also did not produce a Ca^{2+} signal in response to addition of soluble bone matrix. No other bone fractions produced a Ca^{2+} signal in PC-3 cells. It is of interest that bone fractions D3b and D4a contain a number of non-collagenous matrix proteins including osteonectin (SPARC) and osteopontin (OPN), as well as prothrombin. Moreover, antibody LM609 that recognizes the $\alpha_v\beta_3$ integrin, blocks the ability of OPN to trigger a Ca^{2+} transient in PC-3 cells. These studies support a conclusion that bone-matrix proteins play a role in the growth and progression of metastatic prostate cancer, and that prior growth in bone may be associated with development of a bone-matrix-responsive phenotype. © Harcourt Publishers Ltd 2000

INTRODUCTION

Prostate cancer is now the most common type of male malignancy in the USA and the second leading cause of male cancer-related deaths. The number of newly diagnosed cases has doubled in the last decade, and the associated increase in mortality represents a major health issue for males in the USA [1,2]. Carcinoma of the prostate is rarely recognized before the age of 50, and the incidence of the disease increases with subsequent decades [2]. Disease progression follows one of several pathways: peri-prostatic and peri-vesicular penetration, and perforation of the prostatic capsule and invasion along the peri-neural spaces; metastasis to pelvic lymph nodes; metastasis to bones of the pelvis, lumbar spine, thorax and other distal bony sites; and finally, metastasis to soft tissues including the brain. Involvement of the axial skeleton is common, and at autopsy 80% of those with prostatic adenocarcinoma have skeletal metastases [1,3–5]. Because prostate cancer predictably metastasizes to bone, it provides a logical system in which to study the role of bone extracellular matrix proteins in cancer progression and a target for therapeutic intervention.

The composition and architecture of bone provides a uniquely rich environment to support the proliferation of cancerous metastases. The molecular structure of bone consists of fibrils of type I collagen, which constitute approximately 90% of the organic matrix. Collagen fibrils

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may be found in lamellar bone in an ordered array, or in woven bone [6]. Randomly ordered fibrils of collagen, similar to those in woven bone, appear during the osteoblastic response that characterizes prostate cancer invasion of bone. In addition to collagen, bone consists of non-collagenous matrix proteins, serum-derived globular proteins, and sequestered cytokines and growth factors [7]. Some of the non-collagenous matrix proteins include osteonectin (SPARC), osteopontin (OPN), bone sialoprotein and the small bone proteoglycans biglycan and decorin [7]. The identity of the active factors in bone matrix that contribute to accelerated growth and increased metastatic potential of prostate cancer cells has been a recent focus of our group [8]. Along these lines, we previously reported that some solubilized bone fractions, but not all, stimulated growth of prostate cells in soft agar [8].

Ca^{2+} signals provide a convenient measure of the ability of a cell to respond to an extracellular stimulus such as provided by a growth factor [9–11]. Fluorescent Ca^{2+}-sensitive dyes, such as Fura-2, have easily measurable optical responsiveness to Ca^{2+} binding, and rapid kinetics which allow measurement of intracellular Ca^{2+} concentrations in real time. In this study, we measured Ca^{2+} signals in several prostate cancer cell lines in response to soluble bone-matrix proteins. Cell lines included those derived from lymph node, brain and bone metastases.

METHODS

Materials

Coverslip dishes were obtained from MatTek Corp. (Ashland, MA). Fura-2/AM (the acetoxymethyl ester of the Ca^{2+}-sensitive dye, Fura-2) was purchased from Molecular Probes, Inc. (Eugene, OR). Thapsigargin was from Calbiochem Corp. (La Jolla, CA). Thrombin (rat plasma), prothrombin (rat plasma), and adenosine 5′-phosphate (ATP) were purchased from Sigma Chemical Co. (St. Louis, MO). Osteonectin (SPARC) and SPARC polyclonal antibody were provided by Dr Helene Sage, and α_2HS serum glycoprotein was purified in our laboratory as described previously [12]. PC-3 and DU-145 cell lines were obtained from the American Type Tissue Culture Collection (Rockville, MD). Antibody LM609 was provided by Chemicon International Inc. (Temecula, CA).

Fractionation and characterization of bone-matrix proteins

Rat long bones were dissected, crushed, demineralized and extracted as described previously [13]. Non-collagenous proteins were separated into a series of protein fractions using a combination of gel filtration and anion exchange chromatography as described previously [14]. The final collection of proteins fractions used in this study included fractions referred to as ES-2 (small molecular-weight bone proteins from the initial gel filtration step) and a series of fractions in order of increasing anionic charge D1, D2, D3a,b, D4a,b and D5. A general description of the major components of these fractions was described previously and is shown compiled with current data in Table 1 (14). Protein sequence analysis (amino terminal) was routinely performed on a fee for service basis at the Baylor College of Medicine (Houston, Texas) core facility following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or chromatographic separation as described previously [15].

Table 1 Designation and known composition of non-collagenous bone fractions tested in Ca^{2+} imaging experiments

Common fraction name[a]	Known composition
ES1	Large non-collagenous proteins; parent fraction of D-subfractions
ES2	Small non-collagenous proteins, primarily osteocalcin (OCN)
D1	Some soluble collagen fragments; large non-acidic serum proteins
D2	α_2HS-serum glycoprotein
D3a	Unknown
D3b	Osteonectin (SPARC); vitronectin, (pro)thrombin
D4a	Osteopontin (OPN2)
D4b	Bone sialoprotein (BSP); high phosphate-containing (OPN1) dentin sialoprotein
D5	Small bone proteoglycans: decorin and biglycan; bone acidic glycoprotein-75

[a]Demineralized rat bone extracts were resolved into two major fractions termed ES1, ES2 by gel permeation column chromatography as previously described[14]. The ES1 fraction was further fractionated using a cation exchange column eluted with a linear gradient of NaCl, which produced the subfractions designated D1 to D5.

Cell culture

Two human, prostate, cancer cell lines of different metastatic origin were used for this study. PC-3 cells were derived from a patient with bone metastasis, whereas DU-145 cells were derived from a patient with brain metastasis [16,17]. Both cell lines were cultured in Dulbecco's Modified Eagle's Medium-Ham's F12 (1:1; DMEM-F12) medium containing 10% fetal bovine serum (FBS). The well-characterized, androgen regulated cell line LNCaP was also studied, and cultured under well-defined conditions [18]. Two recently isolated cell lines also were obtained from Dr Nora Navone (University of Texas M.D. Anderson Cancer Center) that represent an intermediate cell type between the androgen-independent PC-3 and DU-145 cells and androgen-regulated

LNCaP cells. These cells, A10(PCA2a) and A10(PCA2b) were isolated from bone metastases, but remain fairly slow growing and androgen-responsive [19]. Cells were plated onto coverslip dishes in DMEM-F12 medium containing 10% FBS 2 days prior to the day of the experiment. All cells were subconfluent at the time of the experiments.

Intracellular Ca^{2+} measurements

A single cell Ca^{2+} imaging system (Intracellular Imaging, Inc., Cincinnati, OH) was used to perform intracellular Ca^{2+} measurements as described previously [20]. After removing the medium from the dishes, cells were rinsed with supplemented Hank's Balanced Salt Solution (HBSS), (140 mM NaCl, 4.2 mM KCl, 0.5 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 0.4 mM $MgSO_4$, 0.3 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose, 0.1% FBS, and 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid [HEPES]; pH 7.4). Rinsed cells were loaded with 3 μM Fura-2/AM in supplemented HBSS for 30 min at 37°C. The conditions were chosen to avoid probe compartmentalization and to maximize cytoplasmic dye localization. The loaded cells were incubated for another 15 min with supplemented HBSS alone to allow complete de-esterification of the fluorescent probe. Fura-2 fluorescence was visualized with a Nikon inverted microscope using a Nikon 40X fluor objective. The cells were illuminated with a Xenon lamp equipped with quartz collector lenses. A shutter and filter changer with two different interference filters (340 and 380 nm) were computer controlled. Emitted light was passed through a 430 nm dichroic mirror, filtered at 510 nm, and imaged with an integrating CCD video camera. Four to eight cells were selected for measurement within each visual field. Consecutive frames obtained at 340 and 380 nm were used to obtain a fluorescence ratio (F_{340}/F_{380}), and $[Ca^{2+}]_i$ in each well was calculated from F_{340}/F_{380} by comparison with Fura-2-free acid standards. Individual Ca^{2+} traces shown in the Figures 1–6 are population means derived from simultaneous recording of $[Ca^{2+}]_i$ in the four to eight single cells in a microscopic field. Each experiment was repeated at least three times, and the figures were constructed from representative experiments.

RESULTS

Ability of bone fractions to stimulate Ca^{2+} influx

Table 1 provides the nomenclature used and the known composition of the bone fractions employed in this study. In total, the fractions studied include all non-collagenous proteins present in demineralized bone matrix, as well as some soluble collagen type I present in fractions ES1 (the parent fraction) and fraction D1 (the least acidic fraction eluting first from the DEAE column). They are free of small molecules removed during previous size exclusion chromatography [13]. While many of the components and their elution positions on DEAE were known prior to this study, the identity of the three components of the fraction designated D3b was previously unknown (see below in Results). It is of note that fraction D4a consists primarily of OPN with properties previously described [13], although a more highly phosphorylated form [21] is present in fraction D4b along with bone sialoprotein. In keeping with recently adopted nomenclature in our laboratory, the form of OPN present in fraction D4a, that represents the major OPN found in bone matrix, is called OPN2, and is the isoform produced by bone cells treated with 1,25 $(OH)_2D_3$ [21] and, interestingly, by transformed epithelial cells [22].

In the first series of experiments we performed, soluble bone fractions at various concentrations ranging from 1 to 10 μg/ml, except for ES1, which was also tested at 100 μg/ml, were added to low-density cultures of DU-145 or PC-3 cells loaded with Fura-2 on coverslips. Using the Ca^{2+} imaging instrument, the ability of the bone proteins to trigger a measurable Ca^{2+} transient at any concentration was assessed. Almost all bone fractions failed to raise intracellular Ca^{2+} levels beyond the resting levels (near 50 nM) at any tested concentration, with the exception of two. As shown in Figure 1, addition of protein fractions D3b and D4a rapidly, but transiently, increased intracellular Ca^{2+} concentration to levels approaching 200 nM in PC-3 (panels A and C), but not DU-145 (panels B and D), cells. In all cells tested, subsequent addition of thapsigargin triggered a large Ca^{2+} transient, indicating that all cells were capable of storing and releasing Ca^{2+} from intracellular stores (data not shown). Fraction D4a was known to consist primarily of OPN, but the identity of the active component(s) in fraction D3b was unknown. We next tested the ability of fractions D4a and D3b to stimulate Ca^{2+} transients in three androgen-responsive cell lines including LNCaP (from lymph node) and lines A10 (PCA2a) and A11 (PCA2b) (from bone). Neither fraction elicited a positive result in any of these steroid-responsive lines, regardless of origin. We also tested a second androgen-independent cell line conditioned to growth I bone, C4–2, that represents a sublime of the androgen-dependent LNCaP line. Unfortunately, studies of Ca^{2+} influx in these cells proved problematic because of poor loading of Fura-2 and a tendency to lift off of the cover slips during experimentation. Taken together, it appeared that the ability of the bone fractions to trigger Ca^{2+} transients, and, potentially, growth responses, was unique to the bone-adapted, androgen-independent PC-3 cell line. Interestingly, measurements of stimulation of ^{3}H-thymidine uptake into PC-3 cells showed a dose-dependent stimulation also occurred in response to

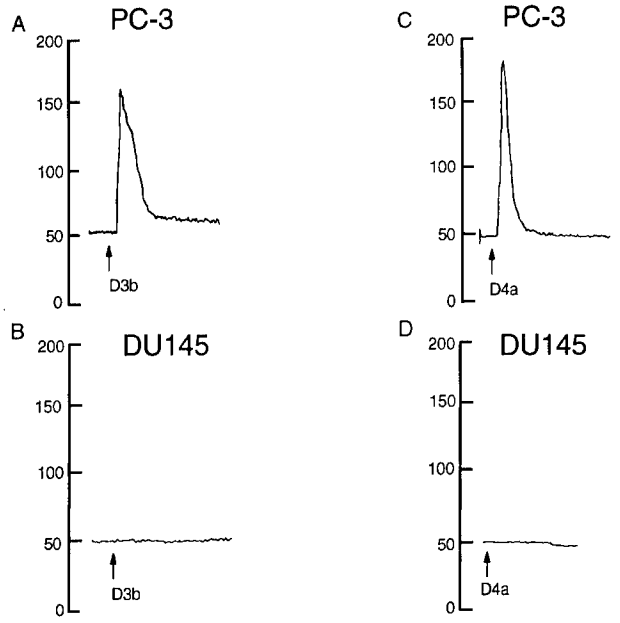

Fig. 1 Ca^{2+} signals in prostate cancer cell lines treated with bone fractions D3b and D4a. Panels A and C depict responses in PC-3 cells and panels B and D depict responses in DU-145 cells. All bone subfractions shown in Table 1 were tested for the ability to stimulate Ca^{2+} transients in both PC-3 and DU-145 cells. No bone fractions, other than those shown in this figure, including D2, which is enriched in α_2HS-glycoprotein, stimulated Ca^{2+} transients (data not shown). As seen, two bone fractions trigger an immediate Ca^{2+} signal in the PC-3 but not the DU-145 cells. Both cell lines respond to the subsequent addition of extracellular ATP (data not shown), indicating they can produce Ca^{2+} transient in response to external stimuli for which they express receptors. The vertical axis represents the intracellular Ca^{2+} concentration (nM). The length of the records shown are 220 s (panels A–C) and 155 s (panel D).

fractions D3b and D4a. Maximum responses were observed at protein concentrations of 5-1 μg/ml, and represented increases in uptake of approximately 2-fold (data not shown).

Extracellular ATP stimulates Ca^{2+} oscillations in both PC-3 and DU-145 cells

Extracellular ATP is known to stimulate Ca^{2+} responses in some cell types [10,23,24]. Because prostate cancer cells

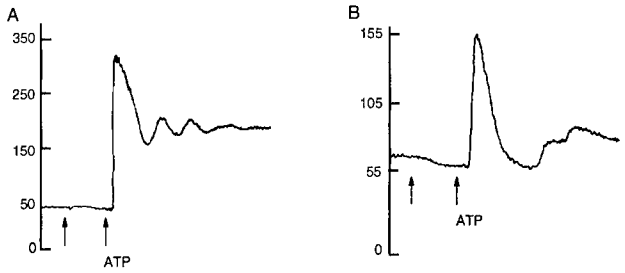

Fig. 2 Ca^{2+} Oscillations in PC-3 and DU-145 cells in response to extracellular ATP. Cell lines were loaded with Fura-2, then Ca^{2+} transients measured in response to the addition of extracellular ATP (0.5 μM). Panel A: development of Ca^{2+} oscillations in PC-3 cells; panel B: similar oscillations in DU-145 cells. As reported in Table 2, no such oscillations were produced by ATP addition to androgen-regulated LNCaP, A10 or A11 cells. The first arrow under the tracing denotes the addition of an inactive bone fraction; the second arrow denotes the time at which ATP was added. The length of the records shown are 440 s.

are often known to display a neurosecretory phenotype, evidenced by secretion of molecules including chromagranin A, we tested the ability of ATP to stimulate Ca^{2+} transients in prostate cancer cell lines including PC-3, DU-145, LNCaP, A10 and A11 (Table 2). As shown in Figure 2, addition of extracellular ATP (0.5 μM) produced Ca^{2+} oscillations in both PC-3 and DU-145 cells, but not in the three androgen-regulated cell lines. The association of these oscillations with secretion has not yet been investigated.

Composition of active bone-protein fraction D3b

Figure 3 shows the separation of active fraction D3b into six distinct protein components by SDS-PAGE. This has now also been accomplished by fast liquid chromatography on a commercial anion exchange column (data not shown). Subjection of proteins electroeluted from preparative gels, or after charge-based chromatographic separation, to amino terminal sequencing demonstrated the identities of some of these proteins. Band 1 is a low abundance band of approximately 80 kDa whose amino terminus was apparently blocked, preventing sequence analysis. Band 2 was identified as prothrombin. Band 3

Table 2 Summary of Ca^{2+} signaling responses in prostatic cancer cell lines[a]

	D2	D3b	D4a (OPN2)	D4b (OPN1, BSP)	Prothrombin	Thrombin	SPARC	ATP	Thapsigargin
PC–3	–	+++	+++	–	+++	+++	++	+++	+++
DU–145	–	–	–	–	–	–	–	+++	++
LNCaP	–	–	–	–	–	–	–	–	+++
A10 (PCA2a)	ND	–	–	ND	–	–	–	–	+++
A11 (PCA2b)	ND	–	–	ND	–	–	–	–	+++

[a]All cell lines were cultured and loaded with Fura-2 as described in Methods
ND = not done

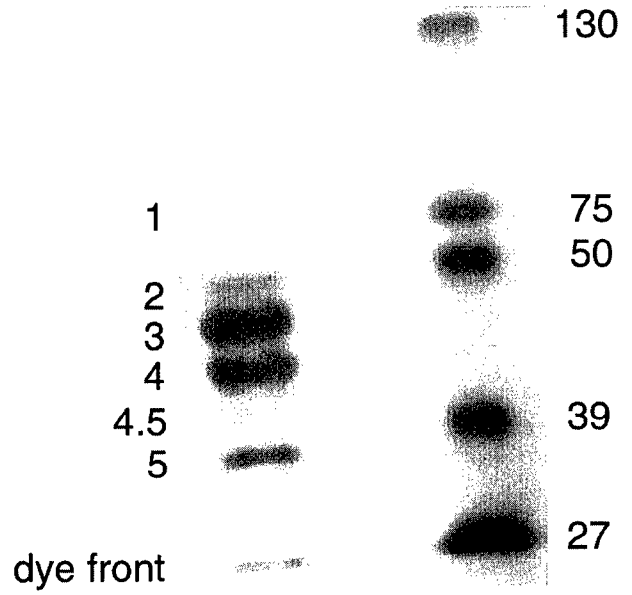

Fig. 3 Electrophoretic and sequencing analysis of proteins found in fraction D3b. The fraction D3b was separated into a series of individual protein bands by SDS-polyacrylamide gel electrophoresis as described in Methods. Following separation, bands were electroeluted and sequenced from the amino terminus. Although several bands demonstrated blocked amino termini (see text), the identity of several protein bands in this fraction was determined. Minor band 1, blocked; band 2, prothrombin; band 3, blocked; band 4, α_2HS serum glycoprotein fragment; band 4.5, SPARC; band 5, blocked.

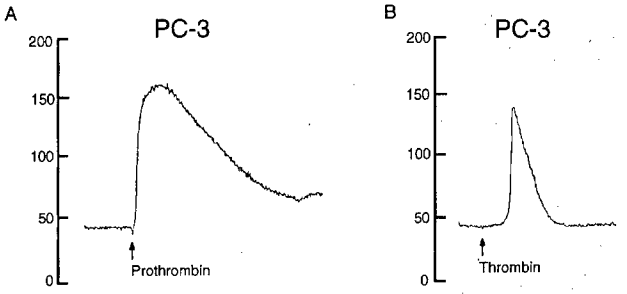

Fig. 4 Recording of Ca^{2+} transients in PC-3 cells treated with prothrombin and thrombin. Ca^{2+} signals in PC-3 cells in response to addition of prothrombin and thrombin were recorded following addition of the (pro)enzyme which was added at the points shown by the arrows. As seen, both proteins triggered a Ca^{2+} transient in PC-3 cells, suggesting that the activation of the thrombin receptor may be at least partially responsible for the Ca^{2+} activation seen with bone fraction D3b. Cell surface conversion of prothrombin to thrombin has been reported (see text). The total lengths of the records shown are 430 s (panel A) and 280 s (panel B).

represents a major component whose N-terminus was also blocked. Band 4 was identified as α_2HS-serum glycoprotein that is greatly enriched in mineralized bone [12]. Band 4.5 (so labeled because we did not detect it immediately due to low abundance levels) corresponds to the bone protein known as osteonectin or SPARC [25]. Band 5 is a 35 or so kDa protein that is also blocked at the N-terminal. It should be noted that non-collagenous bone proteins are commonly blocked artifactually at the N-terminal during their purification from bone, which requires the strong chaotropic agent, guanidinium chloride (William T. Butler, personal communication). This finding does not necessarily mean that these proteins are blocked in vivo.

Ability of prothrombin and thrombin to trigger Ca^{2+} transients in PC-3 cells

The finding of prothrombin in active fraction D3b led us to test the ability of commercially purchased prothrombin and thrombin to stimulate development of Ca^{2+} transients in PC-3 cells. At concentrations between 2 and 5 units/ml, both prothrombin (Fig. 4A) and thrombin (Fig. 4B) stimulated development of a Ca^{2+} transient. Onset of the transient after prothrombin addition was immediate, and consistently larger in magnitude (more sustained) for prothrombin relative to thrombin. A time lag was consistently noted after thrombin addition, and the observed transient was of shorter duration than seen with prothrombin. This pattern was not changed by increasing the thrombin concentration (data not shown), and may be attributed to auto-proteolysis and inactivation. As shown in the pseudo-color time-lapse micrographs in Figure 5, the Ca^{2+} signal induced by prothrombin involved the entire cell cytoplasm. The response to thrombin and prothrombin was restricted to the PC-3 cells, and was not seen in any of the other cell lines that we tested (Table 2).

Activity of other bone proteins in fraction D3b

As shown in Table 2, we also tested the ability of osteonectin, or SPARC, that we found in fraction D3b to trigger Ca^{2+} transients in all five cell lines. Only the PC-3 cells demonstrated a transient in response to addition of SPARC (data not shown). This transient was of lower magnitude than obtained for either D3b, D4a, thrombin or prothrombin. Additionally, antibodies to SPARC did not block the transient induced by fraction D3b. The last identified protein component in fraction D3b is α_2HS-serum glycoprotein, a liver protein enriched in bone. This protein was not able to induce a Ca^{2+} transient in any cell line that we tested, including the PC-3 cells (data not shown). It thus appears that of the protein components in bone fraction D3b known to date, prothrombin is most likely to contribute to the development of the Ca^{2+} signal.

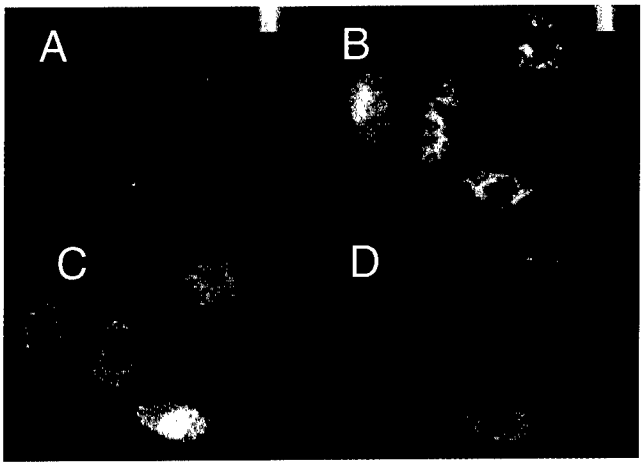

Fig. 5 Pseudocolor images in PC-3 cells treated with prothrombin. The series of panels shown in this figure correspond to the time-dependent Ca^{2+} signals in PC-3 cells treated with prothrombin. The time intervals shown correspond to time periods of 4 s, 1 min 23 s, 1 min 30 s, and 1 min 42 s after addition of prothrombin. Red areas depict the highest Ca^{2+} concentrations (see Fig. 4).

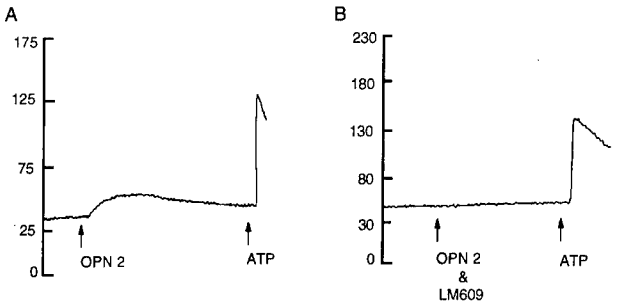

Fig. 6 Ca^{2+} signals in PC-3-Cells in Response to OPN2 and Blocked by Antibody LM609. Panel A: OPN2 isolated from ROS 17/2.8 cells (see text) produces a Ca^{2+} transient in PC-3 cells at concentrations between 5 and 10 μg/ml. The transient shown was produced by addition of 10 μg/ml OPN2. Panel B: pre-treatment of Fura-2-loaded PC-3 cells with antibody LM609 completely abolishes the Ca^{2+} response to addition of 10 μg/ml OPN2. This indicates a role for the $\alpha_v\beta_3$ integrin in the OPN-induced development of a Ca^{2+} transient in PC-3 cells. The total length of the records shown are 405 s.

Involvement of the integrin $\alpha_v\beta_3$ in development of the OPN-induced Ca^{2+} transient

For these studies, we used highly purified OPN that was purified from cultured osteosarcoma cells treated with $1,25(OH)_2D_3$ as described [21]. This OPN isoform that we call OPN2 [21] is typical of that secreted by transformed epithelia [22], and is underphosphorylated relative to some OPN forms produced in bone. As shown in Figure 6, OPN2 (10 μg/ml) produced a Ca^{2+} transient (Fig. 6A) in PC-3 cells that was completely abolished (Fig. 6B) by pre-treatment with the $\alpha_v\beta_3$ blocking antibody LM609 [26]. Non-immune antibody controls had no effect (data not shown). We consistently noted lower responses to OPN isolated from cell cultures relative to OPN isolated from intact bone (compare Fig. 1, panel C to Fig. 6, panel A). The reason(s) for this are presently unknown, but may be due to differences in post-translational modifications that affect OPN function.

DISCUSSION

This line of experimentation was undertaken to assess the role of individual bone-matrix proteins in the development of Ca^{2+} signals in prostate cancer cell lines of various metastatic potential. Both androgen-regulated (LNCaP, A10, A11) and androgen-independent (PC-3, DU-145) cell lines were studied. It is known that Ca^{2+} signals often provide an early measure of the ability of a cell to respond to a growth stimulus [9–11]. No previous study of this nature has been reported, although the ability of bone proteins to stimulate adhesion and/or growth of prostate cancer cells is commonly accepted [27,28]. The exact identities of the active, growth-inducing components present in bone matrix remains unknown, although adhesive interactions involving the $\alpha_2\beta_1$ integrin and bone matrix type I collagen have been reported [28]. These adhesion events are stimulated by TGF-β [28], a bone-sequestered growth factor that was also reported to stimulate proliferation of PC-3 cells [27].

Our approach was to systematically test the ability of bone fractions representing the complete repertoire of non-collagenous proteins present in bone matrix to stimulate development of a Ca^{2+} transients measured by Fura-2 fluorescence. Of interest, we found that the vast majority of proteins in bone had no effect whatsoever on intracellular Ca^{2+} levels in any cell line. In contrast, two bone protein fractions (D3b and D4a) consistently triggered the development of a Ca^{2+} transient in PC-3 cells. This response was unique to the PC-3 cells, and did not occur in either the DU-145 cells nor the three androgen-regulated cell lines. The selectivity of this response to the PC-3 cells is of interest because of the five cell lines tested, the PC-3 cell uniquely represents a highly metastatic, androgen-independent, prostate cancer cell line derived from a human bone metastasis [16]. The DU-145 cell, while also androgen-independent, was derived from a brain metastasis, and does not, therefore, have a history of prior growth adaptation in bone [16]. The LNCaP cell represents an androgen-regulated, less aggressive non-bony tumor [17]. The two new cell lines A10 and A11, while both derived from bone metastases of prostate cancer, are slow-growing and androgen-regulated [19]. They, therefore, may not yet have developed long-term adaptation to growth in bone matrix, and in comparison

to the PC-3 represent an earlier stage of disease progression. These findings suggest that the responses to bone-matrix growth signals, such as Ca^{2+} transients, are likely to represent an adaptive response to long-term growth in the bone environment, such as occurs during transformation to an androgen-independent growth state.

D3b represents a heretofore fairly uncharacterized fraction of non-collagenous bone matrix. We were, however, not completely surprised to find that this fraction stimulated the development of a Ca^{2+} transient in PC-3 cells, because previous studies in our laboratory had shown that this fraction also stimulated growth of prostatic cells in soft agar [8] as well as incorporation of ^{3}H-thymidine into PC-3 cells (not shown). Analysis of the components of bone fraction D3b revealed several surprises, including the presence of prothrombin. This finding was of particular interest because of the growing body of literature demonstrating thrombin receptor (over) expression in malignant cells [29,30], a phenomenon that may be associated with expression of plasminogen activator [30]. Combined with observations that conversion of prothrombin to thrombin can actually occur on the surfaces of cancer cells [31], it is intriguing to speculate that prostate cancer cell activation by bone matrix may involve the thrombin receptor. It is likely in this case that elevations in intracellular Ca^{2+} provide early signals that may accompany activation of growth pathways. Current studies in our laboratory will further investigate this hypothesis.

Other components in bone fraction D3b represent known bone-matrix proteins, including osteonectin (SPARC) and α_2HS-serum glycoprotein [7]. Added in purified form, α_2HS-serum glycoprotein does not produce a Ca^{2+} transient, so it is unlikely to be the active component in D3b. Osteonectin (SPARC) does produce a moderate transient in PC-3 cells, but one that is small relative to that produced by prothrombin. We cannot rule out the possibility that SPARC, or fragments of SPARC, contributes to Ca^{2+} signals in bone-acclimated prostate cancer cells such as the PC-3 cells, but a very recent report [32] suggests that SPARC does not stimulate growth of prostate cancer cells in vitro. Testing of the remaining protein components of D3b will require purification of large amounts of protein, proteolytic cleavage and internal sequencing to determine their identities. These studies are planned.

The finding that protein fraction D4a, which consists almost exclusively of OPN in a low phosphorylated form [21] called OPN2, readily triggers a Ca^{2+} transient was exciting. Previous studies in our laboratory [33] and others [34] have shown that this protein and its mRNA are often up-regulated by transformed epithelial cells including prostate. The more highly phosphorylated form (OPN1) appears to be less active in this regard since fraction D4b, which contains OPN1 and bone sialoprotein, did not readily stimulate a Ca^{2+} transient in PC-3 cells. We investigated this further by purifying pure OPN1 and OPN2 from ROS 17/2.8 cells, under conditions where no protein denaturation occurred. As shown in the results, OPN2 triggered a Ca^{2+} transient in PC-3 cells that was concentration-dependent and maximum at 5–10 μg/ml. Because OPN is known to serve as a ligand to the integrin $\alpha_v\beta_3$ receptor, where it can activate a variety of signaling pathways [35], we tested the involvement of this receptor in PC-3 signaling induced by OPN2. The blocking antibody LM609 [26] was utilized in these studies. When PC-3 cells were pretreated with LM609, the Ca^{2+} transient induced by addition of OPN2 was completely abolished. This is most readily interpreted to mean that OPN2-induced activation of the $\alpha_v\beta_3$ receptor is an essential step in development of a Ca^{2+} signal in PC-3 cells in response to at least some bone-matrix proteins. The relationship between this activation and growth in bone matrix is a subject for further investigation. It is also of note that OPN is a substrate for thrombin cleavage, a finding that has been speculated to have an important physiological role [36].

In summary, the collective findings reported in this manuscript support the conclusion that a subset of bone-matrix proteins can trigger Ca^{2+} signals in bone-adapted cancer cells of prostatic origin. These Ca^{2+} signals are likely to be associated with growth of these metastatic cells in bone. Our studies furthermore suggest that prior growth in bone may be associated with disease progression as reflected by the acquisition of a bone-matrix responsive phenotype.

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Integrin Expression and Usage by Prostate Cancer Cell Lines on Laminin Substrata[1]

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Abstract

During prostate cancer progression, invasive glandular epithelial cells move out of the ductal-acinar architecture and through the surrounding basement membrane. Extracellular matrix proteins and associated soluble factors in the basal lamina and underlying stroma are known to be important regulators of prostate cell behaviors in both normal and malignant tissues. In this study, we assessed cell interactions with extracellular matrix and stromal factors during disease progression by characterizing integrin usage and expression in a series of parental and lineage-derived LNCaP human prostate cancer cell lines. Although few shifts in integrin expression were found to accompany disease progression, integrin heterodimer usage did change significantly. The more metastatic sublines were distinct in their use of $\alpha_v\beta_3$ and, when compared with parental LNCaP cells, showed a shift in α_6 heterodimerization, a subunit critical not only for interaction with prostate basal lamina but also for interaction with the bone matrix, a favored site of prostate cancer metastases.

Introduction

Cancerous prostate cells are regulated in their differentiation, growth, and metastasis by interactions with the surrounding cells and ECMs[4] (1–3). Cell behavior decisions, such as decreasing cell-cell and cell-substrate attachment, and increasing cell motility are accompanied by changes in the expression and/or usage of adhesion receptors, including those of the integrin family (2, 3). Past studies in prostate cancer have focused on quantitation as well as cell surface distribution of integrins (4–8) and on correlating changes in integrin expression with invasive cell behavior (9, 10). The expression level studies have taken two forms: (*a*) cell lines with different metastatic potential have been found to express different levels and subtypes of integrins; and (*b*) within a given cell line, metastatic potential has been experimentally correlated with increases or decreases in levels of integrin expression (9, 11, 12).

Integrin molecular structure, heterodimerization, and intra- and extracellular interactions of integrins with cytoplasmic regulatory proteins and ECM ligands provide tremendous potential for variation among cell types, well beyond that available through quantitative variation in integrin expression level alone. Integrins are themselves heterodimeric molecules, consisting of one α and one β subunit, with at least 20 different combinations already described, many of which differ in their extra- and intracellular binding specificities (13, 14). "Inside-out" regulation of integrin heterodimer activity and subunit partner choices are thought to depend on unique cytoplasmic regulatory protein repertoires, which differ among host cell types (Refs. 15–17 and Ref. 18 and references within). "Outside-in" regulation by integrins, in response to extracellular cues, has also been well studied and has revealed shifts in integrin gene expression as well as changing integrin associations with numerous signaling molecules, including protein tyrosine kinases (focal adhesion kinase and pp60src), serine kinases (protein kinase C, extracellular signal-regulated kinase, c-Jun-NH_2-terminal kinase, and integrin-linked kinase), and lipid intermediates (phosphatidylinositol 3′-kinase and phosphatidylinositol 4,5-kinase; Refs. 14 and 19–21 and the references within). Hence, integrin activity within a given cell is tightly coordinated with its cell cycle, gene expression profiles, differentiation, and cell survival (13). The stroma is a source of key extracellular cues (including soluble growth factors and insoluble matrix proteins) known to modulate integrin-dependent cell functions (22, 23). Although a number of integrin variations during prostate cancer cell progression have been described (5–12), neither modulation of these variations by external factors nor integrin heterodimer usage regulation is well understood.

The LNCaP lineage cell model of prostate cancer progression (24–26) has given us an opportunity to follow coordinated changes in integrin expression, usage, and cell behavior of prostate cancer cells when exposed to different ECM substrata and stromally secreted soluble factors. LNCaP and LNCaP-derived cell lines are unique in that they vary in metastatic potential but share a common genetic background. Previous phenotypic (26) and genotypic (27) char-

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[2] M. E. and T. M. contributed equally to this study.
[3] To whom requests for reprints should be addressed, at Department of Urology, Box 800422, Molecular Urology and Therapeutics Program, University of Virginia Health System, Charlottesville, VA 22908-0422. Phone: (804) 243-6649; Fax: (804) 243-6648; E-mail: Chung@virginia.edu.
[4] The abbreviations used are: ECM, extracellular matrix; FACS, fluorescence-activated cell-sorting; VN, vitronectin; OPN, osteopontin; HGF/SF, hepatocyte growth factor/scatter factor.

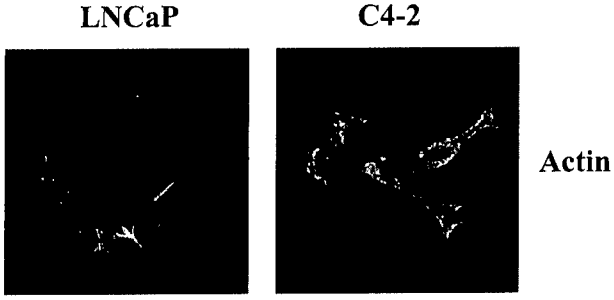

Fig. 1. Actin staining in LNCaP and C4-2 cell lines. Both LNCaP and C4-2 cells show diffuse actin staining and small actin fibers when grown on laminin substrata labeled with phalloidin.

acterizations of these cell lines have revealed their remarkable resemblance to progressing clinical human prostate cancer. We focus here on characterizing interactions between these cancerous prostate cells and their ECM microenvironments, particularly on the ability of cell lines of different metastatic potential to attach, spread, and migrate on laminin, a key protein in both the basement membrane surrounding the acini and in tumors themselves (28). We also examine cell line behaviors on several other matrix components found in bone, a favorite destination for prostate cancer cells following a metastatic cascade (Ref. 29 and the references within). Human prostate tumors disseminated to the bone have been shown to have altered integrin expression, particularly laminin-binding integrin expression, when compared with hyperplastic, benign tumors (11, 12, 30, 31). One integrin heterodimer thought to bind laminin along with VN is $\alpha_v\beta_3$, an integrin that is not expressed in normal prostate tissue but is up-regulated in prostatic adenocarcinoma (11, 32). Likewise, in primary prostate carcinomas, shifts in α_6 integrin subunit expression (and heterodimerization with its β subunit partner) were observed during prostate cancer progression (8). In other tumor cell types, the laminin-binding integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$ have also been linked to acquisition of invasive behaviors (6, 12).

Results

LNCaP Parental and Lineaged Cell Lines Attach to Laminin Using Different Integrin Subunits. Nonmetastatic LNCaP human prostate cancer epithelial cells and their derivative metastatic sublines (C4, C4-2, and C4-2B) readily attached to a common laminin substrate, and all displayed focal contacts and some poorly developed stress fibers, as seen by staining for filamentous actin. The poor development of stress fibers is characteristic of all LNCaP lineage-related cell lines and is not substrate dependent. Representative actin staining in attached LNCaP and C4-2 cells is shown in Fig. 1; C4 and C4-2B cells stained similarly (data not shown).

To identify the integrins used for attachment by the different cell types, parental LNCaP and its derivative C4, C4-2, and C4-2B cell lines were selected (26), and specific, function-blocking integrin antibodies were added to the attachment assays. Although the antibody staining suggested the formation of focal adhesion structures in all cell lines (data not shown), the cells responded differently to the function-blocking antibodies (Fig. 2). Attachment of parental LNCaP cells was best blocked by antibodies against subunits α_6 and β_4, whereas antibodies against these subunits did not effectively block attachment of C4, C4-2, or C4-2B cells, whose attachments were best blocked by antibodies against the intact $\alpha_v\beta_3$ integrin and the subunits α_3 and β_1. Attachments of all four cell lines were also somewhat reduced by antibodies against the subunit α_2.

The Differences in LNCaP and C4-2 Cell Attachment Are Not Likely to Be Due to Differential Expression of Integrin Subunits. FACS analyses were used to determine integrin subunit (α_2, α_3, α_v, β_1, β_3, and β_4) expression levels in LNCaP and C4-2 cell lines (Table 1). Characterization of potential laminin-binding integrin levels by flow cytometry revealed only one difference in expression level (*i.e.,* the α_2 subunit) among the four cell types. Although the expression of the integrin α_2 subunit in C4-2 cells was approximately double that in LNCaP cells, all other integrin receptors were found to remain fairly constant in expression level across all cell lines (Table 1; including C4 and C4-2B; data not shown).

Cell surface expression data were verified by immunoprecipitation of integrin subunits α_3, α_6, $\alpha_v\beta_3$, and β_1 from biotinylated cells of different cell lines (Fig. 3). Similar levels of the α_3 and β_1 subunit were precipitated in all cell lines. The α_3 subunit dimerizes most readily with the β_1 subunit, as seen by immunoprecipitation with either α_3-specific or β_1-specific antibodies (Fig. 3*A*). Although immunoprecipitation with an α_6 antibody coprecipitated β_1 and β_4 subunits from both LNCaP and C4 cells (Fig. 3*B*), the $\alpha_6\beta_1$ heterodimer is not likely to be used for laminin attachment in LNCaP cells because very little inhibition of cell attachment is seen by the β_1 antibody in LNCaP competition experiments (Fig. 2). In comparison with LNCaP, very little β_4 subunit appears to be used for laminin attachment in the C4-2 and C4-2B sublines; the α_6 antibody did not immunoprecipitate as much of the β_4 subunit from the latter two cell lines (Fig. 3*B*), and a function-blocking antibody against β_4 did not inhibit their attachment to a laminin substrate (Fig. 2), as it does for LNCaP. Ratio comparisons, using band intensities on Western blots of α_6-immunoprecipitated β_1 and β_4, reveal a 1:1 ratio of β_1:β_4 in LNCaP cells but show a ratio of 1:0.8 in C4 and 1:0.2 in C4-2 and C4-2B cells.

Although FACS analyses detected both α_v and β_3 subunits in all cell lines, at equivalent surface expression levels, immunoprecipitation with antibody to the $\alpha_v\beta_3$ heterodimer revealed nearly undetectable levels of $\alpha_v\beta_3$ in LNCaP cells while readily detecting the heterodimer in all three derived sublines (Fig. 3*C*). Use of the $\alpha_v\beta_3$ heterodimer does appear to be important for laminin attachment in the three metastatic sublines (but not LNCaP cells) because function-blocking antibody was able to inhibit cell attachment in the sublines (Fig. 2).

The $\alpha_v\beta_3$ Subunit Is Necessary for C4-2, but not LNCaP, Cell Attachment and Migration. Because prostate cancer cells metastasize preferentially to bone, we were particularly interested in the integrin heterodimers known to interact with VN and OPN, two noncollagenous bone matrix proteins. The integrin $\alpha_v\beta_3$ was chosen for attachment and

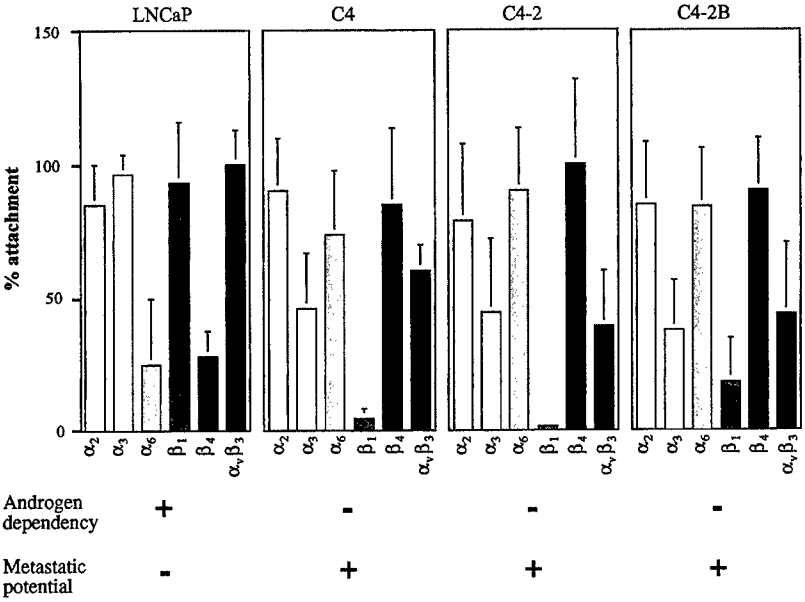

Fig. 2. Inhibition of cell attachment to a laminin substrate using integrin subunit-specific antibodies. LNCaP and the derived sublines C4, C4-2, and C4-2B were preincubated with function-blocking antibodies as indicated. Experimental attachment is shown as a percentage of control, untreated cell attachment, and results are presented as the mean of representative triplicate experiments, with SDs shown as *error bars*.

Table 1 Expression of integrins by FACS analysis

Integrin expression in LNCaP and its more metastatic, derived C4-2 cell line. Values for integrin expression are presented as the mean of two individual duplicate experiments, with the range given in parentheses. An isotype nonspecific antibody was used for control. All experimental fluorescence values are reported as the ratio of the control and specific fluorescence values. Relative values of integrin expression can only be compared for the same antibody on different cells due to differences of antibody affinities for their ligands.

	LNCaP	C4-2
α_2	7.7 (2.6)	13.7 (0.4)
α_3	4.4 (0.6)	3.4 (1.2)
α_6	9.8 (0.4)	10.8 (0.8)
α_v	15.8 (0.8)	14.4 (1.4)
β_1	17.2 (3.2)	15.5 (2.8)
β_3	2.4 (0.8)	2.6 (0.4)
β_4	1.7 (0.6)	1.3 (0.2)

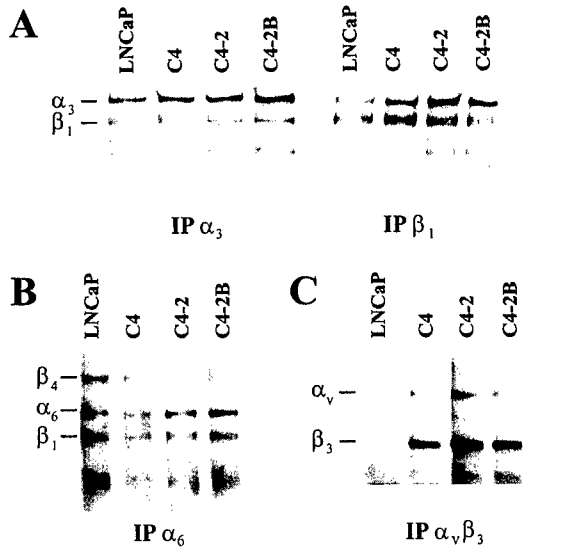

Fig. 3. Immunoprecipitation of biotinylated cell surface integrin isotypes using α_3 and β_1 (*A*), α_6 (*B*), and $\alpha_v\beta_3$ (*C*). Retrieved complexes from each LNCaP, C4, C4-2, or C4-2B cell line were separated by PAGE under reducing conditions, blotted, and visualized with peroxidase-conjugated streptavidin.

migration assays because it is known to interact not only with these two bone matrix proteins but also with laminin (33). LNCaP and C4-2 cells adhered to all three substrata, but only C4-2 attachment could be inhibited with increasing concentrations of antibodies against $\alpha_v\beta_3$ integrin (Fig. 4). At high antibody concentrations of 10 μg/ml, attachments of the metastatic C4-2 cells to all three substrata were reduced by approximately 60%, but no attachment effect was seen for the nonmetastatic LNCaP cells. However, LNCaP attachment could be decreased by using a $\alpha_v\beta_5$ function-blocking antibody (data not shown). The role of the $\alpha_v\beta_3$ heterodimer in cell migration was evaluated using modified Boyden chambers, and the haptotactic responses of each cell line were quantified on laminin, VN, and OPN. Fig. 5 shows the cell migratory behaviors of C4-2 cells on these three bone matrix proteins and that C4-2 cell migration could be inhibited by an $\alpha_v\beta_3$ isotype-specific integrin antibody. LNCaP cells migrated at very low levels on both laminin and VN but did not migrate at all on OPN (data not shown).

Soluble Stromal Factors Induce C4-2, but not LNCaP, Cells to Attach to Laminin. To begin identifying possible regulators of integrin subunit usage and cell behavior in LNCaP and C4-2 cell lines, we tested the effect of stromal factors on cell line interactions with laminin substrata. Cells were treated with conditioned media from primary cultures of the transition or peripheral zone stromal cells of the prostate gland from four different patients with prostatic adenocarcinoma and allowed to adhere for 90 min. Cell spreading was quantified as indicated in "Materials and Methods." Fig. 6*A* shows the differential effects of this soluble paracrine factor on the spread of LNCaP and C4-2 cells. Although all condi-

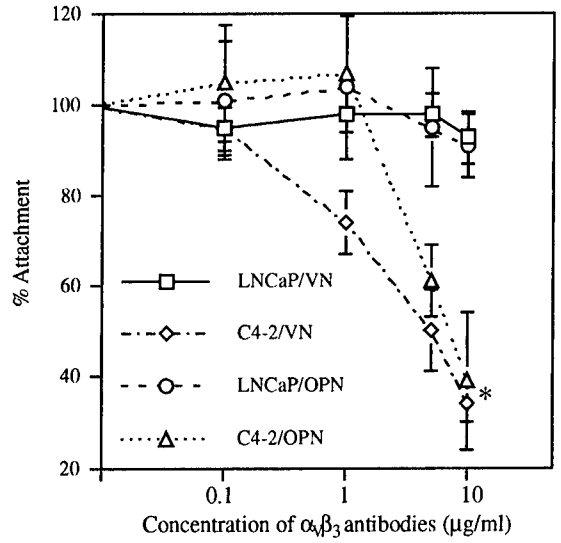

Fig. 4. Antibody-mediated attachment inhibition of LNCaP and C4-2 cells on VN or OPN substrata. Inhibition of attachment is shown with increasing $\alpha_v\beta_3$ antibody concentration expressed as a percentage of control, untreated cell attachment. Values are the mean of two experiments (n = 6), and *error bars* represent SDs. Statistically significant differences from the control were at $P < 0.001$ (*).

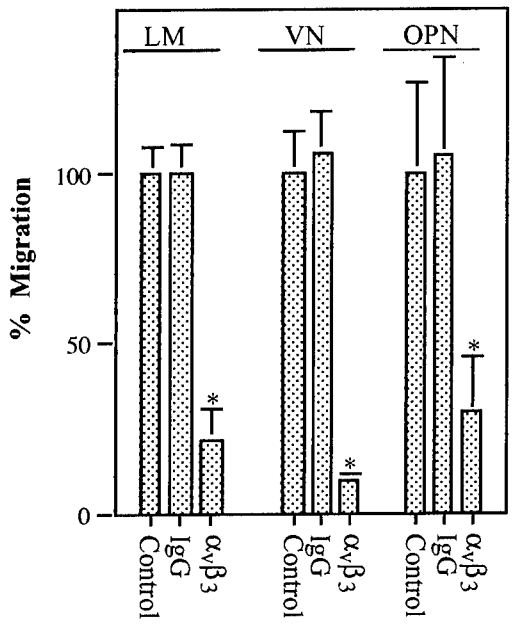

Fig. 5. Migration of C4-2 cells on laminin, VN, or OPN substrata in the presence and absence of 10 μg/ml control or $\alpha_v\beta_3$ function-blocking integrin antibody. Boyden chambers were used for haptotactic assays, and values shown are the average of two experiments (n = 6). *Error bars* represent SDs. Statistically significant differences between $\alpha_v\beta_3$ and the control were at $P < 0.001$ (*).

tioned media caused C4-2 cells to spread more rapidly on laminin, none had any noticeable effect on the spreading of LNCaP cells. No increase in spreading was seen for either cell line when treated with conditioned media from mouse fibroblastic cells (Sw3T3 cells; data not shown).

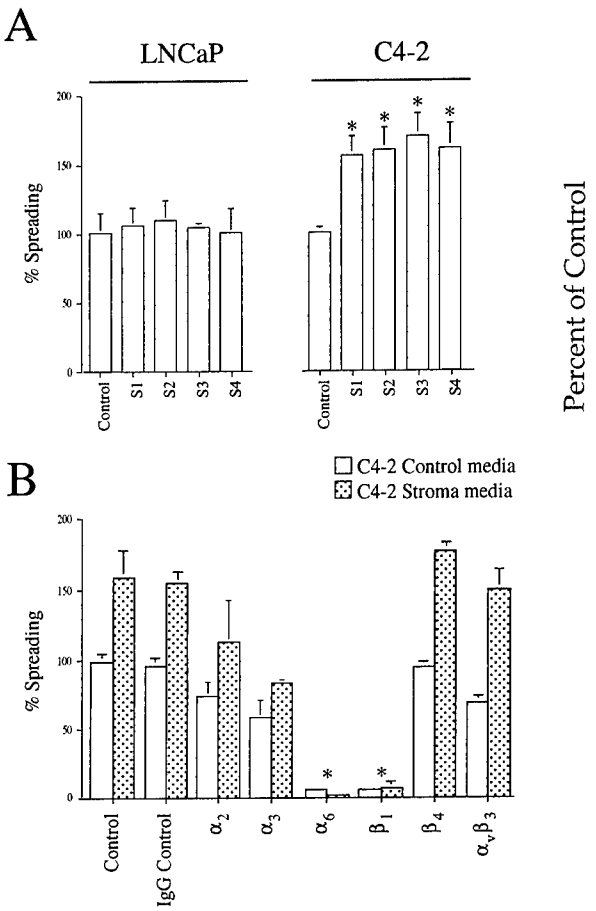

Fig. 6. LNCaP and C4-2 cell attachment to laminin substrata after treatment with stromal cell-conditioned media and/or integrin isotype-specific antibodies. *A*, LNCaP and C4-2 cell attachment responses to conditioned media from four primary prostate stromal cell cultures (three different patients). All percentage attachment values are normalized to the behavior of control, LNCaP, and C4-2 cells that were not treated with conditioned media (after 90 min, 25% of C4-2 untreated control cells had attached, compared with only 6% of LNCaP untreated control cells). *B*, comparison of C4-2 cell attachment percentages after treatment with stromal cell-conditioned media in the presence or absence of function-blocking, integrin isotype-specific antibodies. All experiments were repeated six times. Statistically significant differences between treated and untreated cells were at $P < 0.001$ (*).

The effects of conditioned media could be reversed using integrin isotype-specific, function-blocking antibodies. Fig. 6*B* shows that function-blocking antibodies to both α_6 and β_1 inhibit cell spreading in both control and stromal cell-conditioned media-treated cells. Function-blocking antibodies against the α_2, α_3, and $\alpha_v\beta_3$ integrins also were able to block 20–50% of the increase in cell spreading induced by conditioned media. Quantification of integrin cell surface expression by FACS analyses (Fig. 7) did not reveal any change in receptor availability between control and stromal cell-conditioned media-treated cells, thus the observed variation in cell response to external regulation is unlikely to be based on changing integrin profiles but instead appears to be based on the improved efficiency of C4-2 cells in use of specific integrin isotypes for cell spreading in the presence of prostate stromal cell-conditioned media.

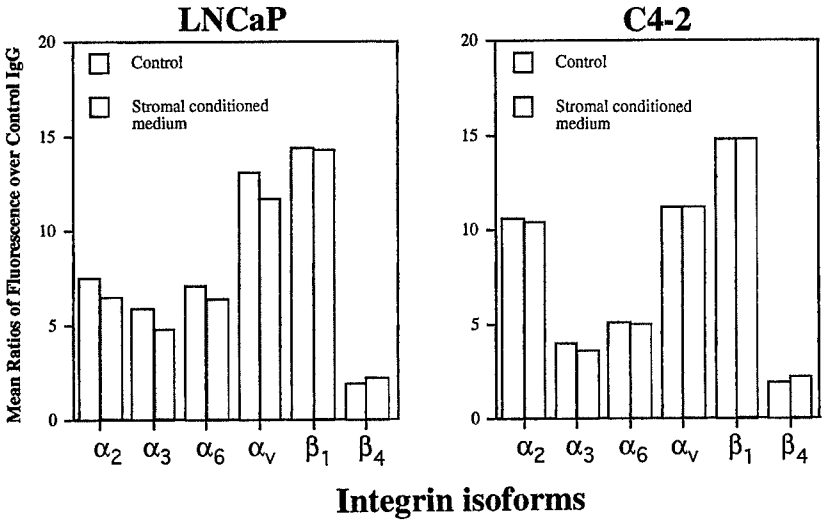

Fig. 7. FACS analysis of cells treated with stromal cell-conditioned media. Surface expression of integrin subtypes after 90 min of treatment with stromal cell-conditioned media is shown. No change in the integrin surface expression is evident.

Table 2 Comparison of previously reported integrin usage during prostate cancer progression and the usage found in the LNCaP progression model.

Integrin heterodimer	LNCaP	C4-2	Normal to carcinoma (Ref. no.)
$\alpha_3\beta_1$	+	++	Stable (8, 34)
$\alpha_6\beta_1$	+	++	Stable (5, 6, 8, 34)
$\alpha_6\beta_4$	++	–	Decreases (5, 7, 33)
$\alpha_v\beta_3$	–	+	Increases (11)

Discussion

During prostate cancer progression, changes occur not only in the microenvironments of cells but in the cells' reception and interpretation of cues from these environments. We have focused here on shifts in integrin receptor expression and usage accompanying cancerous progression in the prostate and occurring in response to cues, such as stromally secreted factors. Although we did find expression levels of the integrin subunit α_2 to be elevated in metastatic cell lines, overall, the usage of integrin subunits varied more strikingly than did expression level between cell lines and varied in response to exposure to stromal factors. The integrin usage we detected in the LNCaP model system correlated well with previously published immunohistochemical staining for integrin expression in patient specimens (Table 2; Refs. 5–8, 33, and 34) and added to a number of past *in vitro* studies showing differences in integrin heterodimer expression among cultured normal, neoplastic, and prostate carcinoma cells (10, 34–37). Our results may also help clarify previous studies of integrin expression in various epithelial carcinomas, whose results have conflicted with one another, and may have implications in prostate cancer cell homing to the skeleton, along with preferential survival and proliferation in the bone microenvironment.

Integrin subunit partner choice repeatedly appeared to affect cell migratory and adhesive behaviors in the absence of shifts in subunit expression levels. For example, the partner choice of the integrin α_6 subunit varied between cell lines, whereas the integrin β_1 and β_4 subunit expression levels remained constant among lines of very different invasive behaviors. LNCaP cells attached to laminin primarily with $\alpha_6\beta_4$, whereas cells in the more invasive C4-2 subline attached with $\alpha_3\beta_1$ and $\alpha_v\beta_3$ (Figs. 2 and 3). This shift in α_6 usage fits with previous studies, in which $\alpha_6\beta_1$ and $\alpha_6\beta_4$ were both found in normal prostate cells, but β_4 subunit expression was lost in carcinomas (5, 6, 8). Because the α_6 integrin subunit preferentially associates with β_4, it is believed that a reduction in β_4 subunit expression results in a relative increase in the formation of the $\alpha_6\beta_1$ heterodimer (38). The varied pattern of integrin heterodimerization in these cell lines could be due in part to differential expression of α_6 subunit isoforms. The integrin α_6 subunit exists as two isoforms, α_{6A} and α_{6B} (34, 38–42), both of which are expressed in LNCaP cells (34). Although we do not yet know the specific α_6 isoform expressed by C4-2 cells, overexpression of the α_{6A} isoform is known to increase $\alpha_6\beta_1$ heterodimerization as well as overall cell motility, tumorigenicity, and invasion (12).

$\alpha_6\beta_4$ use declined in C4-2 cells, in conjunction with an increased use of $\alpha_6\beta_1$, $\alpha_v\beta_3$, and $\alpha_3\beta_1$ (Fig. 2). Unlike $\alpha_6\beta_4$, which is associated with stable, hemidesmosomal cell attachment sites and appears to restrict cell migration, $\alpha_6\beta_1$ and $\alpha_3\beta_1$ are both involved in the formation of dynamic focal contacts important for cell locomotion (12). Prostate cell lines able to form invasive tumors in immunocompromised mice have previously been shown to have increased expression of the $\alpha_6\beta_1$ heterodimer (10, 12, 36, 43), and antibodies against $\alpha_6\beta_1$ are able to inhibit invasion. Like Vafa *et al.* (36), we too found the $\alpha_6\beta_1$ heterodimer to be more involved in cell spreading than static cell attachment (Figs. 2 and 6), but the role of this heterodimer in metastatic cell interpretation of environmental cues, such as matrix and secreted factors, requires further study. The $\alpha_3\beta_1$ integrin is likely to have both direct and indirect effects on cell motility because of its bidirectional interactions with the matrix. The ability of $\alpha_3\beta_1$ to alter laminin chains and overall basement membrane architecture (44, 45) is particularly suggestive, given that proteolytic cleavage of laminin can drive cells from static adhesion to active migration (46, 47). It is interesting to note in this

context that oncogene-transformed rat prostate cells express elevated levels of both laminin type I and the $\alpha_6\beta_1$ integrin (36, 43).

An additional integrin heterodimer implicated in increased metastatic potential and tumorigenicity is $\alpha_v\beta_3$ (48–50). Although not frequently found in epithelial cells, $\alpha_v\beta_3$ is common to a number of bone-receding metastases, including prostate and breast carcinomas (10, 11, 51). In the LNCaP model system, $\alpha_v\beta_3$ was similar to $\alpha_3\beta_1$ in that its individual subunits were expressed at all stages of cancerous progression (that is, in all cell lines), but the assembled, functional heterodimers were only detectable in the more metastatic cell lines C4, C4-2, and C4-2B (Table 1; Fig. 3). Although such differences in integrin usage have been noted before between very different cell lines with different metastatic potentials, this is the first study we know of that reveals such shifts in integrin usage between cells with common genetic backgrounds but different *in vivo* metastatic potentials.

Two possible consequences of $\alpha_v\beta_3$ heterodimer usage in the metastatic LNCaP sublines are (*a*) preferential relocation to the bone and (*b*) increased cell survival/suppressed cell death. Integrins are likely to be involved in both the establishment of prostate cell anchorage to the bone endothelium and its surrounding matrix and the transmission of multiple cues from the cells' microenvironments supporting cell survival and proliferation. Not only do C4-2 cells, cells known to preferentially relocate to bone (26), increase their use of the $\alpha_v\beta_3$, but we show here that they use this integrin to migrate on OPN, a key component of bone matrix. $\alpha_v\beta_3$ has also previously been shown to support migration on VN, another dominant component of bone matrix (52, 53). Regardless of the role of $\alpha_v\beta_3$ in binding metastatic cells to the bone matrix, this integrin heterodimer is a good candidate regulator of cell survival in the absence of cell adhesion. Although loss of appropriate adhesion is normally a cue for apoptosis, human breast cancer cells are able to use $\alpha_v\beta_3$ to inhibit p53 activity and suppress the bax death pathway (54). Likewise, $\alpha_v\beta_3$ has been shown to regulate cell proliferation in prostate epithelia (55).

Integrin regulation of prostate epithelial proliferation is likely to involve interactions between integrins and growth factor receptors. Such receptors are used by cells to interpret positive and negative growth factor and cytokine signals from surrounding stromal cells (Refs. 1 and 56 and the references within), and they do so through common signaling cascade components (for example the small GTPases), which are also important for integrin signaling and activation. There is evidence that the two types of surface proteins may associate directly and preferentially with one another (55). In the context of changing integrin usage [such as that observed between LNCaP and C4-2 cells or reported previously *in vivo* (see Table 2)], preferential associations between the growth factor receptors and the changing integrin heterodimers could have serious consequences for the cells' responses to environmental cues. Indeed, Fig. 6 shows that C4-2 and LNCaP cells do respond differently to soluble factors in media from prostate stromal cells, with only C4-2 cells showing increased spreading on laminin substrates after stromal media treatment.

We investigated the roles of $\alpha_6\beta_1$ and $\alpha_v\beta_3$ integrins in response to stromal cues by adding function-blocking antibodies against these integrins to C4-2 cell cultures before and after treatment with prostate stromal cell-derived conditioned media. The dramatic increase in C4-2 laminin spreading after treatment with such conditioned media was relatively unaffected by $\alpha_v\beta_3$ function-blocking antibodies, whereas antibodies against either α_6 or β_1 completely obliterated spreading on laminin both before and after stromal cell-conditioned media treatment (Fig. 7), a result in agreement with the work of Vafa *et al.* (36) on c-erb B2/neu-transformed rat prostate epithelial cells. The identities of the responsible soluble factors (cytokines, growth factors, or others) behind the $\alpha_6\beta_1$-specific response remain to be determined, although one candidate growth factor, which is found in the conditioned media, is the HGF/SF.[5] Although purified HGF/SF has the ability to stimulate prostate cancer cell spreading and migration when placed on ECM substrata, HGF/SF is only one of many such factors secreted by the prostate stromal cells. Whether growth factor receptors on the C4-2 cells interact directly with nearby $\alpha_6\beta_1$ integrins after stimulation by a stromally secreted growth factor is also unknown. Stimulated receptors could also signal the integrins indirectly through intracellular cofactors, such as focal adhesion kinase (62).

In summary, use of a lineage-derived LNCaP cancer cell progression model has allowed us to compare the integrin expression levels, heterodimer usage, and cell behaviors in cells sharing a common genetic background but differing in their metastatic potentials on different matrices and in the presence or absence of stromal factors. We have found that although integrin expression levels do not change markedly among the cell lines (with the exception of an increase in collagen binding α_2 expression), integrin heterodimer usage does change. In particular, the androgen-independent and invasive LNCaP derivative C4-2 subline shows marked differences in its use of $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, and $\alpha_v\beta_3$ when compared with that of the androgen-dependent and noninvasive parental LNCaP cells. Although all cells attached to laminin, VN, and OPN matrices, only the more invasive and metastatic C4-2 cells were able to migrate on OPN. C4-2 cells were also unique because of their response to prostate stromal cell-derived factors. The striking increase in the spreading of C4-2 cells on laminin after treatment with stromal factors could be completely obliterated by the addition of function-blocking antibodies against α_6 or β_1, but not against α_2, α_3, β_4, or $\alpha_v\beta_3$. Because C4-2 cells were found to increase usage of $\alpha_6\beta_1$ but decrease usage of the $\alpha_6\beta_4$ heterodimer, additional studies are called for to characterize this shift in heterodimer usage and its direct and/or indirect effects on cell behavioral and survival responses to matrix and stromal environmental cues. Such future studies promise to have profound implications for control of metastatic human

[5] M. Edlund, T. Miyamoto, R. A. Sikes, R. Ogle, G. W. Laurie, M. C. Farach-Carson, C. A. Otey, H. E. Zhau, and L. W. K. Chung. Regulation of cell adhesion in prostate cancer cell lines by hepatocyte growth factor, manuscript in preparation.

prostate cancer cell dissemination, proliferation, and survival in the skeleton.

Materials and Methods

Cell Culture, Antibodies, and ECMs. LNCaP cells and their more metastatic sublines, C4, C4-2, and C4-2B (25), were grown in T-media (Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal bovine serum. Primary cultures of prostate stromal cells were derived from the tissue surrounding prostatic adenocarcinomas, as described by Ozen *et al.* (57). Conditioned media were prepared by adding fresh media without serum when cells reached 80% confluence and removing it 48 h later. Laminin-1 was purified from Engelbreth-Holme-Swarm tumors according to Davis *et al.* (58), based on the protocol of Kleinman *et al.* (59). OPN-2 was purified as described by Devoll *et al.* (60). VN was purchased from Promega (Madison, WI). Antibodies to integrin subunits α_2, α_3, α_6, $\alpha_v\beta_3$, β_1, β_3, and β_4 were all obtained from Chemicon (Emecula, CA). Vinculin antibody (V9131) was obtained from Sigma (St. Louis, MO), and all secondary-conjugated antibodies were obtained from Jackson Immunochemicals (West Grove, PA).

Immunofluorescent Confocal Microscopy. Cells were seeded onto glass coverslips coated with 50 μg/ml laminin-1. For immunocytochemistry, cells were allowed to spread, fixed in 3% formaldehyde, permeabilized in 0.2% Triton X-100, and stained using either FITC-labeled phalloidin to label filamentous actin or antivinculin antibody (V9131) to detect focal adhesions. Texas Red-conjugated goat anti-mouse secondary antibodies were obtained from Jackson Immuno Research (Bar Harbor, ME). Cells were mounted on glass coverslips with gel-Mount (Biomedia Corp.), and images were acquired using a laser-scan confocal microscope 410 (Carl Zeiss, Minneapolis, MN).

Flow Cytometry Analysis. Cells below 70% confluence were detached from tissue culture plates and suspended as single cells using a brief treatment of 10 mM EDTA and 20 mM HEPES buffer (pH 7.4) in T-media. The EDTA was neutralized with $CaCl_2$ and $MgSO_4$, and the cells were washed again with T-media containing 0.1% BSA. A total of 2.5×10^5 cells were used for each preparation. Cells and primary antibodies (30 μg/ml) were incubated for 60 min at 4°C, washed, and further incubated with secondary FITC-labeled goat anti-mouse (30 μg/ml) antibody for an additional 60 min at 4°C. After three brief washes, 1×10^4 cells were analyzed for fluorescence using a FACScan (Becton Dickinson, San Jose, CA). Cells treated with isotype-specific immunoglobulins served as controls. For both cell types, the relative fluorescence intensity was expressed as the increase over background fluorescence. Data points were presented as the mean of two independent experiments, with a range in parentheses (Table 1).

Substrate Adhesion, Attachment, and Migration Assays. Cell attachment and competition assays were performed as described by Vafa *et al.* (36). Assay plates were precoated with laminin, VN, or OPN by overnight incubation at 4°C and subsequently blocked with heat-inactivated BSA for an additional 4 h at room temperature. For adhesion assays, cells were trypsinized with 0.2% trypsin/2% EDTA in PBS (pH 7.2), suspended in T-media for titration to single cell suspension, and centrifuged briefly. Resuspended cells were then held in adhesion media [T-media with 20 mM HEPES (pH 7.4), 7 mM EDTA, and 0.1% BSA] for 5 h at 37°C and 5% CO_2 to ensure reexpression of integrins on the cell surface. After preincubation, $CaCl_2$ and $MgSO_4$ were added to neutralize EDTA. Cells (5×10^3) in 100 μl of serum-free media were added to each well and allowed to attach for 6 h at 37°C. Triplicate cultures were prepared for each condition. After culture, cells were washed twice in PBS and stained using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (61, 62) or visually counted.

For the attachment assay with or without stromal cell-conditioned media, cell lines were grown to confluence, trypsinized, and replated (1:8) on tissue culture dishes, where they were allowed to grow for another 2 days before being lifted and treated briefly with 10 mM EDTA and 20 mM HEPES buffer in T-media. After neutralizing the EDTA with $CaCl_2$ and $MgSO_4$, the cells were washed with T-media containing 0.1% BSA. Cells were finally held in unconditioned or stromally conditioned media for 10 min, placed on laminin-coated dishes, allowed to adhere for 90 min, and then fixed in formaldehyde. Visible lamelopodia or fillopodia categorized a cell as positively spread. Each cell line was scored for the percentage of spread cells, and all values were normalized to that of control cells that had not been subjected to treatment with conditioned media. At 90 min, untreated LNCaP cells spread on laminin and VN at percentages of 5–10% and 45%, respectively, while at the same time point, untreated C4-2 cells spread on laminin and VN at percentages of 25–35% and 40%, respectively.

Haptotaxis was assayed in triplicate using modified Boyden chambers with an 8 μm pore size [Becton Dickinson (Bedford, MA) or Corning (Acton, MA)]. PBS (100 μl) containing laminin (50 μg/ml), VN (50 μg/ml), or OPN (20 μg/ml) was placed on the underside of the porous membrane and chambers were pre-incubated at 4°C overnight. PBS (100 μl) alone served as a negative control. On the second day, chambers were assembled with serum-free T-media containing 0.1% BSA. Cells (5×10^4) were added to the upper chambers and incubated at 37°C, 5% CO_2 for 16 h. Cells were then fixed with 2% parafomaldehyde and stained with crystal violet. Cells remaining in the upper chamber were removed with a cotton swab. Cells that had migrated were counted using light microscopy; for each condition, 10 randomly chosen fields of cells were counted, and the results were presented as an average ± SD. Migrated control cells were counted at densities of approximately 100 cells/mm^2.

Cell Surface Biotinylation. Integrins on cells surfaces were biotinylated as described previously (63). Briefly, cells were washed in PBS and incubated with 500 μg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL) for 30 min at room temperature. Cells were then washed in 50 mM glycine and PBS before they were lysed [20 mM HEPES (pH 7.4), 150 mM NaCl, 1% NP40, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin]. Cell extracts were precleared with protein A/G-agarose beads (Oncogen Science, Cambridge, MA) for 1 h at 4°C and spun at 10,000 rpm for 30 min. Integrin subunits were retrieved by immunopre-

cipitation. Integrin subunit-specific antibodies (200–500 μg/ml) were incubated with the cell lysate for 1 h at 4°C, and immunocomplexes recovered using protein A/G-coated agarose beads. Complexes were analyzed by nondenaturing 7.5% PAGE and electroblotting. After transfer, filters were blocked in 5% milk for 1 h at room temperature. Filters were then incubated with horseradish peroxidase-streptavidin, and proteins were detected using enhanced chemiluminescence (Amersham, Piscataway, NJ).

Statistical Analyses. Where applicable, data were analyzed using Excell or QuickTTest, for determination of mean, SD, and parametric statistics (paired *t* test).

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Type I collagen-mediated proliferation of PC3 prostate carcinoma cell line: implications for enhanced growth in the bone microenvironment.

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ABSTRACT

Prostate cancer is the second leading cause of male cancer-related deaths in the United States. Interestingly, prostate cancer preferentially metastasizes to bone. Once in the bone microenvironment, advanced prostate cancer becomes highly resistant to therapeutic modalities. Several factors, such as, extracelluar matrix components, have been implicated in the spread and propagation of prostatic carcinoma. The prostate cell line, PC3, adhere and spread on collagen I to a greater degree than on fibronectin (FN) or poly-L-lysine (PLL). Flow cytometry analysis reveals the presence of the $\alpha 1$, $\alpha 2$ and $\alpha 3$ collagen binding integrin subunits. Antibody function blocking studies reveal that PC3 cells can utilize $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins to adhere to collagen I. Cells plated on collagen I exhibit increased rates of proliferation over cells plated on FN or tissue culture plastic. Additionally, cells plated on collagen I show increased expression of cyclin D1, a molecule associated with progression through G1 phase of the cell cycle. Inhibitor studies point to a role for phosphatidylinositol 3-kinase (PI3K), map kinase (MAPK) and p70 S6 kinase in collagen I-mediated PC3 cell proliferation and cyclin D1 expression. Type I collagen may facilitate the colonization and growth of metastatic prostate tumor cells in the bone microenvironment.

INTRODUCTION

The American Cancer Society estimates that in the year 2000, 180,400 cases of prostate cancer will be diagnosed, and 31,900 American men will succumb to the disease, making prostate cancer the second leading cause of male-related cancer deaths in the United States (Greenlee et al., 2000). While prostate specific antigen (PSA) screening has assisted clinicians to diagnose prostate cancer in its early stages, some patients still present with metastatic lesions in the bone. Additionally, more than 70% of patients with advanced disease exhibit bone metastases (Chiarodo, 1991). These patients experience significant pain due to spinal cord compression and pathological bone fractures. Most significantly, metastatic prostate cancer is often refractory to most therapeutic modalities, representing a major obstacle in treating patients with advanced disease. Often, palliative treatment is the sole option in these cases. Since a prominent feature of prostate cancer is its ability to colonize and thrive in skeletal tissue, better understanding of the complex biology of prostate cancer bone metastasis will allow for the development of novel and effective treatments for patients with advanced metastatic disease.

Bone is a dynamic tissue that is constantly being remodeled under the direction of various systemic hormones and locally derived cytokines and growth factors. Remodeling is the result of the coupled action of two bone-specific cell types. The hematopoietic-derived osteoclasts degrade bone, while the stromally derived osteoblast is responsible for directing new bone formation (Canalis et al., 1991; Mundy, 1999). Osteoblast activity results in the deposition of numerous growth factors and extracellular matrix molecules (ECM) in mineralized bone tissue, and remodeling cycles liberate a variety of chemotactic substances that may influence the homing to and initial seeding of bone by metastatic prostate cells (Hullinger et al., 1998; Lang et al., 1995). Additionally, remodeling activity may make available various mitogens, such as insulin-

like growth factor I (IGF-I) and basic fibroblast growth factor (bFGF), that possibly potentiate the proliferation and survival of prostate cancer cells in bone (Gleave et al., 1992; Iwamura et al., 1993). Clearly, the skeletal microenvironment is an inviting target for prostate cancer cell metastasis.

In addition to the supply of growth factors, the ECM of skeletal tissue also can impact skeletal metastasis of prostate cancer. The organic component of the bone ECM consists primarily (>95%) of type I collagen (Termine, 1990). The remaining non-collagenous component consists of molecules such as bone sialoprotein, osteopontin, thrombospondin, vitronectin, fibronectin, osteocalcin and osteonectin. Previous studies show that these non-collagenous proteins either by themselves or in various combinations can promote prostate cancer cell adhesion and growth (Jacob et al., 1999; Koeneman et al., 1999; Lecrone et al., 2000; Thalmann et al., 1999). However, given its abundance in the bone matrix, the role collagen I may play in prostate cancer bone metastasis has yet to be elucidated. Previous studies demonstrate that collagen I serves as an adhesive substrate for numerous cancer cells and may influence adhesion and retention of various metastatic cells in skeletal tissue (Klein et al., 1991; Kostenuik et al., 1996; Kostenuik et al., 1997; Ridley et al., 1993). Type I collagen, therefore, is an attractive candidate molecule capable of influencing homing and adherence of prostate cells to bone.

Prostate cancer bone metastasis involves the complex interplay of prostate tumor cells and the skeletal microenvironment. Knowledge of this interplay is important in designing effective therapies to treat patients with advanced prostatic skeletal disease. In the present study, we provide evidence that the major component of bone ECM, type I collagen, may serve as a mediator of prostate tumor bone metastasis. Employing the human prostate carcinoma cell line, PC3, we identified type I collagen as a major adhesive substrate for PC3 cells and demonstrate

that type I collagen serves as a permissive substrate for enhanced PC3 cell proliferation. Inhibitor studies suggest that the phosphatidylinositol 3-kinase (PI3K) pathway is the major signaling molecule responsible for PC3 cell proliferation on a type I collagen matrix. These results suggest a role for collagen I in facilitating prostate cancer cell bone metastasis by enhancing both cellular attachment and proliferation.

RESULTS

PC3 Cell Adhesion to Type I Collagen

Initial experiments were carried out to investigate the adhesive properties of PC3 cells to the predominant bone component, type I collagen. Fibronectin (FN), a major integrin adhesive substrate for numerous cell types, was used as a comparison matrix for PC3 cell adhesion. Adhesion results were normalized to poly-L-lysine (PLL), a receptor independent adhesive substrate. Adhesion of cells to plates coated with BSA served as the negative control. Serial dilutions were performed with the test substrates and the concentration that supported maximal adhesion (data not shown) was used for all subsequent experiments. As shown in fig. 1, PC3 cells rapidly adhere to type I collagen, exhibiting maximal adhesion at 30 min with no significant increase at the 60 min time point. In contrast, the number of PC3 cells adhered to FN was roughly half that bound to the PLL control at 30 min. A slight increase in the number of adhered cells was noted at the 60 min time point.

Morphological examination of PC3 cells revealed an interesting difference between the binding of cells to FN compared to type I collagen. Cells plated on PLL, while bound to the tissue culture plate, remained round and did not spread (fig 2 a,d) at 30 and 60 minutes. Adherence to FN resulted in neglible spreading activity at 30 min (fig 2 b) and only at 60 min did cell spreading commence (fig 2 e). In contrast, cells plated on type I collagen exhibited a fully spread morphology at both 30 and 60 min (fig 2 c,f).

To determine the identity of the integrin subunits responsible for type I collagen binding, PC3 cells were screened for the presence of collagen binding integrins. Specific anti-integrin antibodies were incubated with PC3 cells and were analyzed by flow cytometry. Results demonstrated the presence of all three known collagen-binding integrins; $\alpha 1$, $\alpha 2$ and $\alpha 3$ on the surface of PC3 cells (fig 3). Function blocking monoclonal antibodies were employed in

adhesion assays to examine the role of the various integrins in PC3 cell binding to type I collagen. Cells were pre-incubated with a maximal concentration of 10μg/ml of antibody for one hr and then were plated onto tissue culture wells coated with 1.0μg/cm^2 type I collagen. Again PLL served as the control test substrate to which data were normalized. Control mouse IgG at matched concentrations did not negatively influence binding to collagen I. An anti-α1 function blocking antibody decreased PC3 cell adhesion to collagen I by 54.9% (fig 4). Additionally, function blocking monoclonal antibodies against α2β1 and α3 decreased cell binding by 86.1% and 89.3% respectively (fig 4).

PC3 cellular proliferation on type I collagen

ECM molecules not only act as adhesive substrates, but they also influence numerous aspects of cell physiology, including cellular proliferation. PC3 cells were plated onto either uncoated wells or wells coated with FN or type I collagen and incubated overnight in 1% serum. Cells were shifted to serum-free medium to begin the experiments and proliferation was measured using the MTS reagent. As shown in fig 5, PC3 cells exhibited a 55.1% ($p<0.05$) increase in proliferation, measured at 24 hr, over that of cells plated on uncoated control wells. In contrast, PC3 cells plated on FN only showed a modest increase of 8.13% over the uncoated control. Results of the MTS assay were confirmed with manual cell counting (data not shown). At 48 hours, the proliferation of PC3 cells only increased to 59.7% over control on type I collagen.

Engagement of integrins by ECM molecules activates numerous signal transduction pathways. To define signaling molecules that regulate PC3 cell proliferation mediated by type I collagen, cells were exposed to inhibitors of specific signaling molecules thought to play a role in

integrin-mediated cellular proliferation were employed. Treatment of PC3 cells with 25μM of PD98059, a selective inhibitor of the MAP kinase pathway, resulted in a decrease in PC3 proliferation of 19.1% at 24.0 hrs and 36.1% at 48.0 hrs compared to untreated control (fig. 6). Use of rapamycin, which results in the inactiviation of p70 S6 kinase, at a concentration of 25nM decreased proliferation by 30.6% at 24.0 hrs and 46.2% at 48.0 hrs compared to that of the untreated control sample (fig 6). The specific inhibitor of the PI3-kinase enzyme, LY294992, at a concentration of 25μM, inhibited PC3 proliferation by 50.4% at 24.0 hours and 63.6% at 48.0 hours (fig 6) making it the most potent of these three inhibitors tested.

As another marker of proliferation, the regulation of cyclin D1 expression was investigated in PC3 cells plated on the various substrates was investigated. Cyclin D1 is an important modulator of the cell cycle whose expression is rapidly up-regulated as cells enter the cell cycle (Ekholm et al., 2000). Integrin engagement by molecules in the ECM also is capable of inducing cyclin D1 up-regulation and cell cycle progression (Roovers et al., 2000). PC3 cells were grown to confluency and shifted to serum-free medium for 48 hours to reduce basal levels of cyclin D1 expression before plating on test substrates. This treatment had no discernable effect on cell viability (data not shown). As shown in fig. 7, minimal expression of cyclin D1 protein was observed in cells following serum starvation. Cyclin D1 expression levels increased to the greatest extent in the cells plated on type I collagen. This increase correlates with the increase in proliferation exhibited when cells are plated on type I collagen. The levels of cyclin D1 expression observed in the cells plated on PLL and FN is consistent with the basal level of cell proliferation that occurred in the PC3 cell line. The levels of cyclin D1 protein expression were decreased in cells that were pretreated for one hr with the inhibitors, an observation that correlates with the levels of inhibition observed in the proliferation assay. The greatest inhibition occurred in cultures pre-treated with the PI3-kinase inhibitor, LY294992, and rapamycin the p70

S6 kinase inhibitor. Treatment with these two inhibitors results in a ratio of 1.3 when levels of cyclin D1 are compared to the actin load control. PD98059 inhibit to a lesser extent, again in accord with the proliferation data.

DISCUSSION

Skeletal tissue represents an inviting target for the metastatic spread of prostate cancer cells. The rich supply of growth factors located in the bone microenvironment potentiates the metastatic spread and growth of prostate tumor cells. The skeletal ECM also may influence prostate metastasis by promoting attachment, growth and subsequent survival of prostate cancer cells in bone. The major component of the skeletal ECM is type I collagen. We, in agreement with a report by Kostemuik and colleagues (Kostenuik et al., 1996; Kostenuik et al., 1997), propose that type I collagen serves as an adhesive substrate for PC3 prostate carcinoma cells, potentially regulating the attachment and colonization of metastatic cells to bone. In addition, we provide evidence that type I collagen stimulates the proliferation of PC3 cells. These results provide a potential important insight into the interaction between prostate tumor cells and the bone ECM that may be useful in the development of novel and effective treatments.

Here, we report that the PC3 cell line, which is derived from a human bone metastasis (Kaighn et al., 1979), rapidly adheres to type I collagen in an integrin-dependent manner. PC3 cells showed maximal adhesion by 30 minutes to type I collagen with the majority of the cell population exhibiting a spread morphology. In comparison, PC3 cells adhered to a lesser extent to FN, a common ECM component, and cells bound to FN showed little spreading at 30 and 60 minutes. The propensity of PC3 cells to adhere and spread on type I collagen offers an explanation for the osteotropism observed in metastatic prostate tumor cells.

Cells utilize integrin molecules to bind extracellular matrix components such as type I collagen (Hynes, 1992). The integrin pairs responsible for collagen binding are $\alpha 1\beta 1$, $\alpha 2\beta 1$ and $\alpha 3\beta 1$ (Heino, 2000). Flow cytometry analysis revealed the presence of all three type I collagen binding integrin sub-units on the surface of PC3 cells. These results differ from those of Kostemuik et al (Kostenuik et al., 1997), who reported that PC3 cells expressed low levels of the

α1β1 and α3β1 integrins. There are a number of possible explanations for this discrepancy. For example, there may exist subtle phenotypic differences in the particular PC3 cell clones employed in the two studies. Alternatively, the different monoclonal antibodies used in performing the flow cytometry experiments and differential-binding affinities may account for the discrepancies observed in the levels of potential integrins on the surface of the PC3 cells. Although there were differences in the α1β1 and α3β1 integrin levels, the levels of α2β1 on the PC3 cells were high in both studies. To identify the integrin receptors necessary for PC3 cell binding to type I collagen, we undertook a series of antibody blocking experiments. Our results demonstrated that both the α2β1 and α3β1 integrins were able to serve as major type I collagen-binding receptors for PC3 cells. The α1β1 also is utilized by PC3 cells but to a much lesser extent. Our results expand the observations of Kosternuik et al (Kostenuik et al., 1997). who suggested that PC3 cells bind to type I collagen solely through the α2β1 integrin. Again these differences may be the result of differences in the phenotypes of cells used and differences in the blocking antibodies utilized. Both studies, however, are in agreement with the conclusion that PC3 cells readily adhere to type I collagen through an integrin-dependent mechanism, minimally through the α2β1 integrin receptor. These studies provide intriguing data to support a model whereby metastatic prostate cells arrest in skeletal tissue by binding to type I collagen through the α2β1 integrin. Further support of this hypothesis comes from studies showing that TGF-β, which is abundant in bone, increased binding of PC3 cells to a type I collagenous matrix by up-regulating the α2 integrin subunit (Kostenuik et al., 1997). Additionally, the over expression of the α2 integrin sub-unit in rhabdomyosarcoma cells conferred them with the ability to metastasize to bone (Chan et al., 1991).

Metastatic cells must be capable of arresting in distant organ sites, and also must be able to thrive in their new environment. We provide evidence that type I collagen, the major ECM component in skeletal tissue, provides a signal for metastatic prostate cell proliferation. PC3 cells plated on type I collagen proliferate at rates roughly 50% over that of cells growing on plastic. These results underscore the importance of the ECM in modulating cellular functions. Normal cells are dependent on the adhesion to ECM components to survive and proliferate in response to a variety of mitogenic stimuli, whereas transformed cells are marked by their ability to thrive in the absence of contact to ECM (Giancotti et al., 1999). Given that independence, transformed cells do remain competent to respond to ECM components (Varner et al., 1996). For example, contact with various ECM molecules affects expression of genes encoding certain pro-metastatic proteins, such as various proteases, in numerous tumor cell types (Boudreau et al., 1999). These results suggest that type I collagen may not only serve as an adhesive substrate for prostate cancer cells in bone but also may contribute to the outgrowth of metastatic cells in skeletal tissue.

Integrin engagement by ECM components results in the activation of numerous signaling molecules culminating in a variety of cellular processes, such as cellular proliferation (Giancotti et al., 1999). To investigate signaling pathways that modulate PC3 proliferation on type I collagen we used a variety of inhibitors to measure the contribution of candidate signaling molecules. Our results indicate that the major signaling molecule that mediates the type I collagen proliferative effect appears to be PI3-kinase. The PI3-kinase enzyme phosphorylates certain inositol lipids leading to the activation of numerous downstream signaling molecules. The activity of PI3-kinase modulates various cellular processes including proliferation, gene expression and cell survival (Vanhaesebroeck et al., 1997). The specific PI3-kinase inhibitor LY249442 abrogates type I collagen-mediated proliferation in PC3 cells by 63.6% at 48 hrs,

suggesting that PI3-kinase activity drives proliferation of PC3 cells on type I collagen. The second signaling molecule investigated was p70 S6 kinase. Activation of the p70 S6 kinase signals for G1 to S phase progression and subsequently cell proliferation (Thomas et al., 1997). The use of the inhibitor, rapamycin, which results in decreased activity of the p70 r6 kinase diminished proliferation to a lesser extent than LY249442. The possibility exists that p70 S6 kinase may function downstream of PI3-kinase as reported previously. The other major proliferative pathway activated by integrin ligation is the MAP kinase pathway (Roovers et al., 2000). Our results provide evidence that this pathway also impacts type I collagen directed proliferation, but to a much lesser extent than the PI3-kinase inhibitor. MAP kinase activity also has been shown to act downstream of PI3-kinase, therefore the possibility exists that the pathways converge downstream (Krasilnikov, 2000).

As another marker of cell proliferation we examined the protein expression of cyclin D1 protein. Cyclin D1 expression is up regulated as cells prepare to progress from G_o to G_1 where it complexes with cyclin-dependent kinase 4 and 6. The concerted activity of the cyclin-cdk complex drives progression through the various phases of the cell cycle (Ekholm et al., 2000). The expression of cyclin D1 is controlled in part by integrin binding to ECM (Zhu et al., 1996). We observed a modest, but measurable, level of cyclin D1 expression in PC3 cells. Cells plated on type I collagen exhibited an increased rate of cyclin D1 protein up-regulation as compared to cells plated on other matrices. Treatment of cells with the inhibitor LY249442 show a marked decrease in expression of cyclin D1 protein, providing further evidence for the role of PI3-kinase in signaling for cellular proliferation. Rapamycin decreased the level of cyclin D1 protein expression to half that of PI3-kinase that corresponds to the level of decrease in cell proliferation. The MAP kinase inhibitor demonstrated a neglible effect on Cyclin D1 expression. Thus cyclin

D1 levels in cells plated on type I collagen were up regulated in what appears to be a PI3-kinase-dependent and p70 S6 kinase-dependent pathway that mirrors levels of cell proliferation.

In the present study we provide evidence that type I collagen, a major constituent of the skeletal ECM, serves as an adhesive substrate supporting proliferation of the bone metastasis-derived prostate tumor cell line, PC3. The collagen mediated proliferative signal appears to be mediated by PI3K. Our results suggest a role for type I collagen in potentiating the growth of prostate tumor cell growth in the skeletal microenvironment.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents

PC3 cells, a human prostatic carcinoma cell line derived from a bone metastasis, were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, VA) and 100 U/mL of penicillin and 100 μg/ml of streptomycin (Life Technologies, Rockville, MD). The inhibitors LY290042, rapamycin and PD98059 were obtained from Calbiochem (La Jolla, CA). Blocking antibodies against integrin α1 (clone FB12), α2β1 (clone BMA2.1) and α3 (clone ASC-1) were purchased from Chemicon (Temecula, CA). Human fibronectin and rat-tail type I collagen were purchased from Collaborative Biomedical Products (Bedford, MA).

Cell Adhesion Assay

Concentrated stocks of rat-tail type I collagen were diluted to indicated concentrations in 0.02 N glacial acetic acid and coated onto Maxisorp 96 well plates (Nunc, Naperville IL) for 1 hr at room temperature. Human fibronectin was diluted in 1X PBS (pH 8.0) and also coated for 1 hr on Maxisorp 96 well plates. Plates were washed twice with 1X PBS (pH 8.0) and blocked with 3% heat-denatured bovine serum albumin (BSA) for 1 hr at 37°C followed by two washings with 1X PBS (pH 8.0). Cells were harvested with 0.5 mM EDTA and collected by centrifugation. Pelleted cells were washed twice in serum free RPMI 1640, resuspended in serum-free RPMI 1640/0.1% BSA (pH 7.4), and plated at a concentration of 2.5×10^4 cells per 100μl. The assay was terminated at indicated time points by washing wells twice with serum free RPMI 1640. Cell adhesion was quantitated with the CellTiter 96® AQ_{ueous} Assay (Promega, Madison WI) which is composed of solutions of a novel tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS),

and an electron coupling reagent phenazine methosulfate (PMS). MTS bioreduction by cells into a formazan product was measured at 490 nm on a Dynex MRX miroplate reader (Chantilly, VA). Cell adhesion was represented as a percentage of cells adhering to test substrate compared to the number binding to a poly-L-lysine control substrate. Antibody blocking studies were performed as above with the exception of a 1 hr pre-incubation with 10μg/ml of antibody before plating onto wells coated with test substrates.

Flow Cytometry

1 x 10^6 cells were harvested as described above and incubated with appropriate concentrations of primary antibodies in 1X PBS/0.1% BSA on ice for 30 mins. Negative control antibodies consisted of appropriately matched isotypes at the same concentrations as primary antibodies. Cells were washed twice with cold 1X PBS/0.1% BSA and counter-stained with appropriate species-specific secondary FITC-conjugated antibodies 30 min on ice. Cells were washed twice with cold 1X PBS/0.1% BSA, resuspended in 2.5% paraformaldehyde and stored at 4°C until analysis on a FACSCalibur Flow Cytometry System (Beckton Dickinson, Franklin Lakes, NJ).

Cell Proliferation Assay

Cells were harvested with 0.5mM EDTA/1X PBS and collected by centrifugation and washed twice with 1X PBS. Cells were plated onto either standard 96 well tissue culture plates, BIOCOAT collagen I or fibronectin (Becton Dickinson, Bedford, MA) 96 well plates at a concentration of 3.5 x 10^4 cells per well in RPMI 1640 containing 1% fetal calf serum. Following an overnight incubation, cells were washed 1X with serum free RPMI 1640 and cultured in serum free RPMI 1640 containing various concentrations of inhibitors for indicated time periods. Cell proliferation was quantitated by measuring the conversion of MTS tetrazolium to formazan as described above. Data was plotted as a percentage of MTS

conversion by cells plated on un-coated control wells for each time point. MTS results were confirmed with trypan blue and manual cell counting.

Cyclin D1 Assays

Cells were harvested with 0.5mM EDTA and collected by centrifugation and washed twice with 1X PBS. 1.5×10^5 cells were plated on previously coated 4-well plates in serum free RPMI medium. At the end of one hr, cells were lysed in SDS sample buffer (62.5 mM tris-HCL, pH6.8, containing 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5mM β-mercaptoethanol) and sonicated for 5 one-second bursts. Equal cell equivalents were separated on 10% SDS polyacrylamide gels and electroblotted to a nitrocellulose membrane. After an initial blocking period of 1 hr at room temperature with 5% milk, blots were probed overnight at 4° C using a 1:1000 dilution of anti-cyclin D1 antibody (BD Pharmingen, San Diego, CA). After incubation with appropriate secondary peroxidase conjugated antibody, blots were developed with Pierce SuperSignal chemiluminescent substrate (Rockford, IL). Blots were stripped and re-probed with a 1:2000 dilution of an anti-actin (Sigma, St. Louis, MO) to control for differenced in loading. Numerical data were obtained by setting the ratio of the time 0 cyclin D1 integrated density values (IDV) to time 0 actin IDV levels to one and comparing ratios of other treatments points to this value. IDV values were obtained using Scion Image program (Scion Corporation, Frederick, MD).

Statistical Analysis

Data and statistical analyses were performed on GraphPad Prism and InStat software programs. Experimental data was subjected to one way ANOVA with subsequent Tukey-Kramer post test.

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FIGURE LEGENDS

Figure 1. Adhesion of PC3 cells to fibronectin and type I collagen. Harvested PC3 cells were plated onto tissue culture wells coated with the indicated concentrations of adhesive substrates. After the indicated incubation period the plates were washed and adhesion measured by conversion of MTS substrate by adherent cells. Values are presented as a percentage of cells binding to test substrate compared to cells binding to a poly-L-lysine control substrate. The data represent the mean and standard deviation (S.D.) of at least three independent experiments with four replicate wells.

Figure 2. Morphological characterization of PC3 cell adhesion to matrix components. PC3 cells were plated onto tissue culture wells coated with 10μg/ml PLL (a,d), 2.5μg/cm^2 FN (b,e) or 1.0μg/cm^2 type I collagen (e,f).

Figure 3. Flow cytometry analysis of integrin surface expression on PC3 cells. Harvested PC3 cells were stained with monoclonal antibodies recognizing either α1 (clone FB12) integrin, α2β1 (clone BMA2.1) integrin or the α3 (clone ASC-1) integrin. The clear histogram represents cells stained with the indicated anti-integrin antibody. The shaded box represent cells stained with appropriate isotype control antibodies.

Fig. 4. Blocking of integrin-mediated adhesion of PC3 cells to type I collagen. Harvested PC3 cells were pre-incubated with 10μg/ml of anti-integrin antibodies for 1 hr in suspension and plated on tissue culture wells pre-coated with 1.0μg/cm^2 type I collagen. After the indicated incubation period the plates were washed and adhesion measured by conversion of MTS

substrate by adherent cells. Values are presented as a percentage of cells binding to test substrate compared to cells binding to a poly-L-lysine control substrate. The data represent the mean and S.D. of at least three independent experiments with four replicate wells. (***$p<0.001$)

Figure 5. PC3 cell proliferation on type I collagen. PC3 cells were harvested and plated on tissue culture dishes pre-coated with FN or type I collagen overnight in 1% FCS. The next day cells were shifted to serum-free media and MTS conversion was measured at 24 and 48 hrs. Data were plotted as a percentage of MTS conversion by cells plated on un-coated control wells for each time point. (*$p<0.05$).

Figure 6. PC3 cell proliferation inhibition assay. PC3 cells were harvested and plated on tissue culture dishes pre-coated type I collagen overnight in 1% FCS. The next day cells were shifted to serum-free media containing the either LY294002 (25μM), Rapamycin (25nM) and PD98059 (25μM). Data presented as percent decrease in cell proliferation of treated wells compared to untreated wells at the same time point. ($p<0.001$, at all time points examined)

Figure 7. Western blot analysis of cyclin D1 protein expression in PC3 cells. Harvested cells were suspended in serum-free media for 1 hr with either no treatment or with the following treatments: LY294002 (25μM), rapamycin (25nM) and PD98059 (25μM). After treatment, cells were plated on type I collagen (1.0μg/cm^2) coated 4-well tissue culture plates for 1 hr and lysed with SDS reducing sample buffer. Equal cell equivalents were separated on 10% SDS-PAGE gels, transferred to nitrocellulose and immunoblotted with antibodies against cyclin D1 or beta actin. The integrated density values of individual bands were determined by analysis with the

Scion Image program and normalized to beta actin expression. One of three independent experiments shown.

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Figure 1

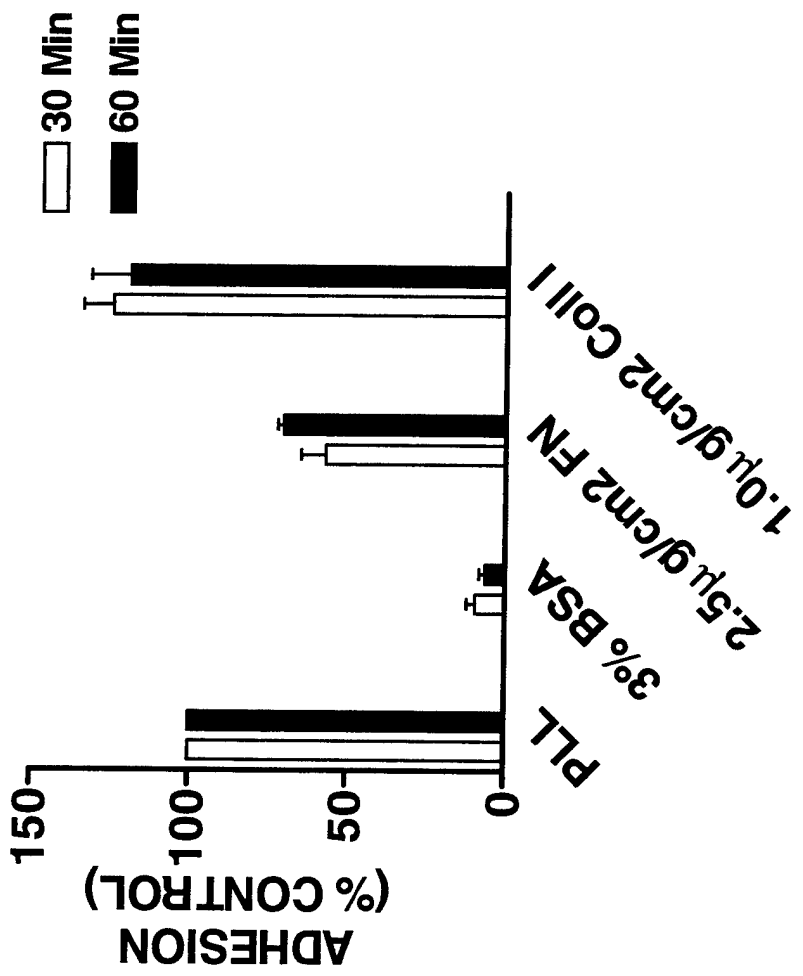
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Figure 2

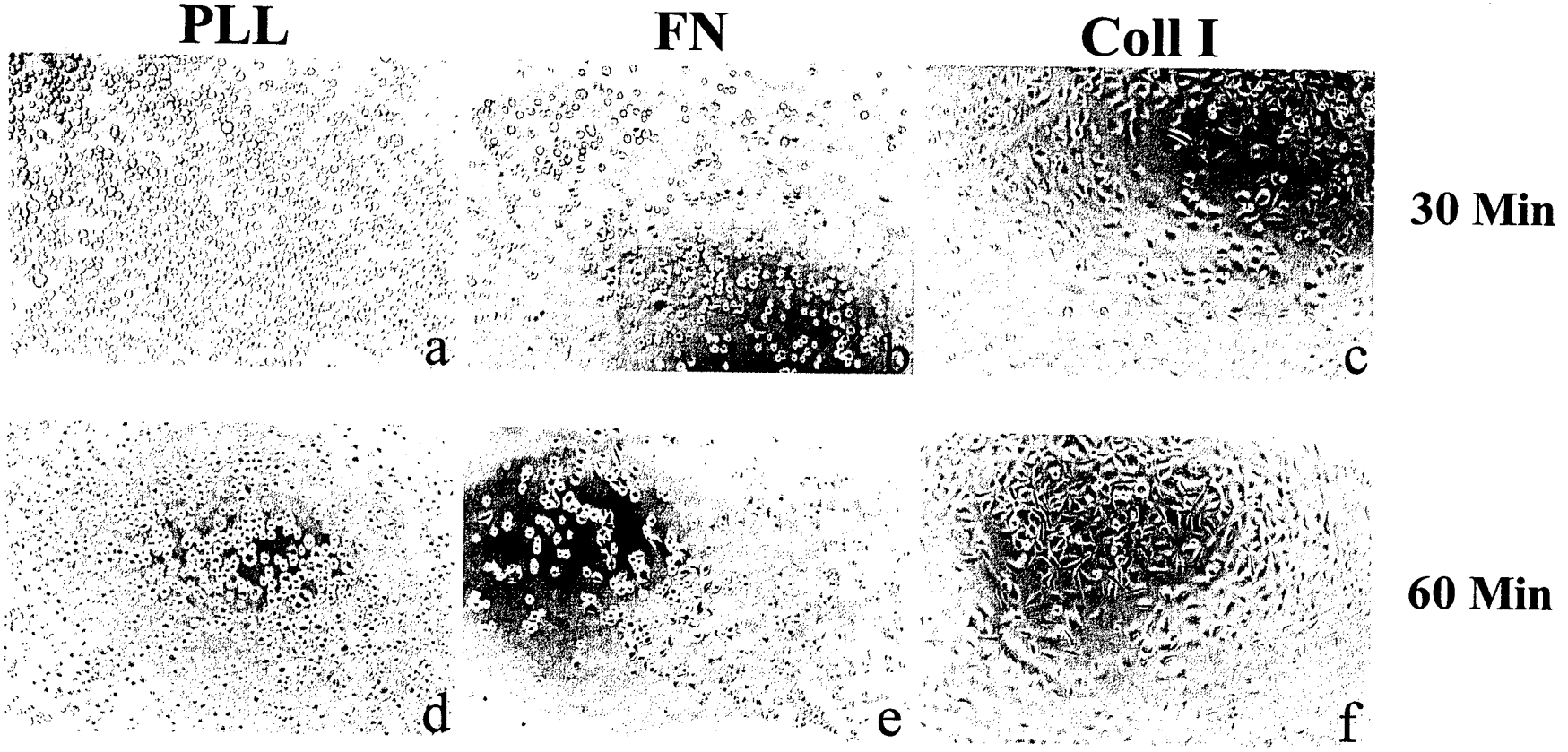

Figure 3

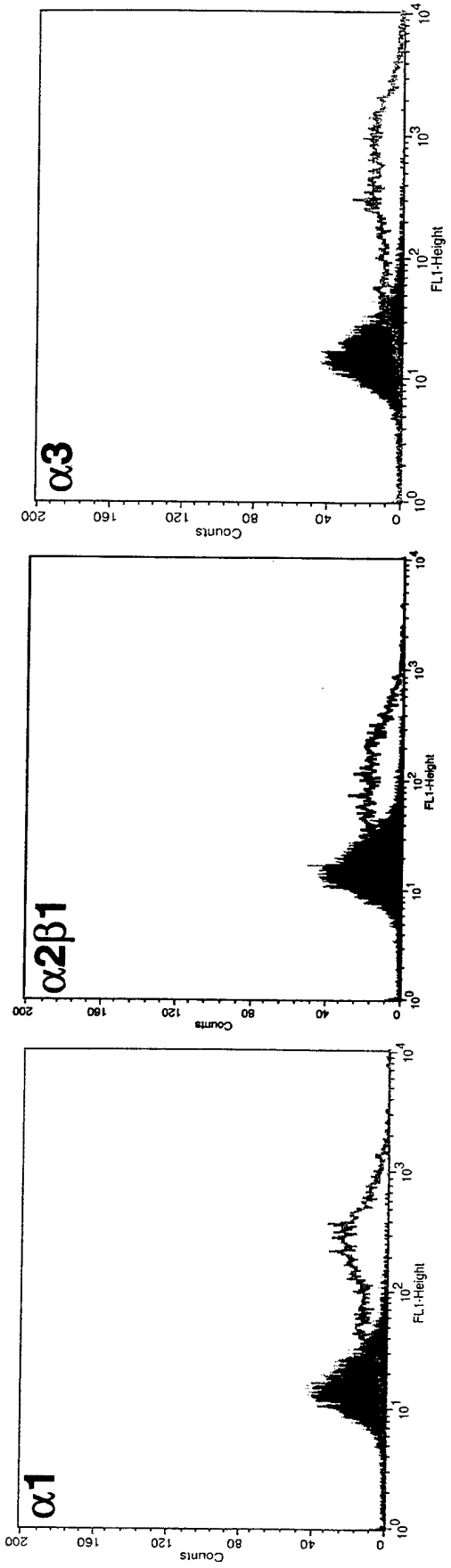
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Figure 4

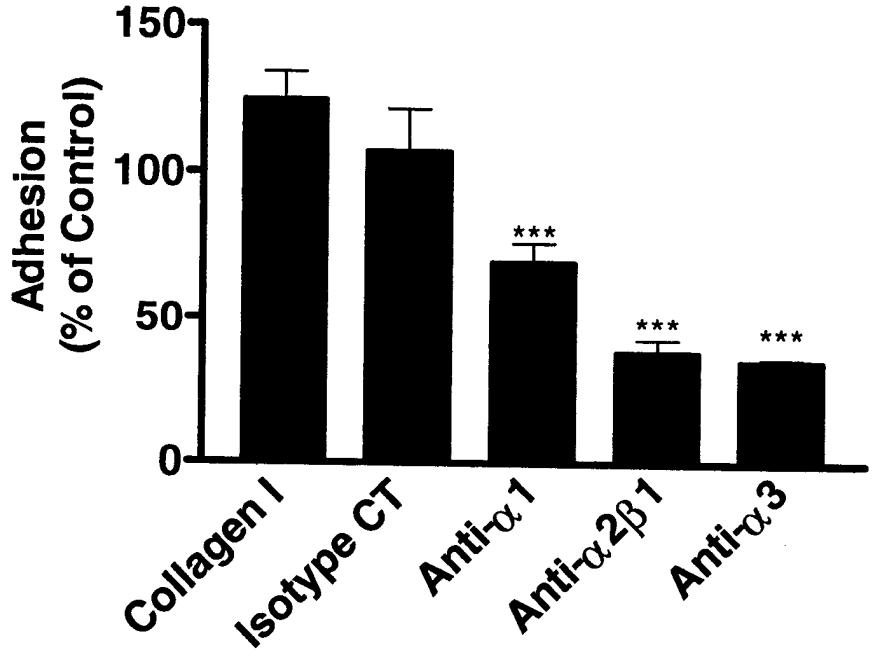
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Figure 5

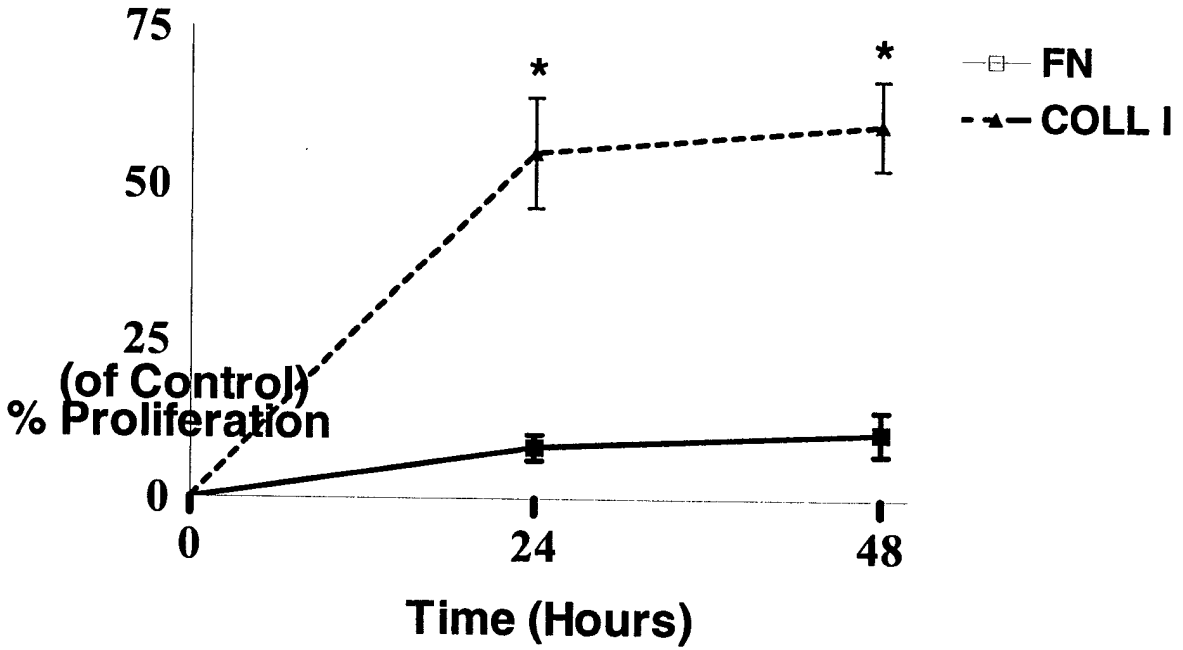
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Figure 6

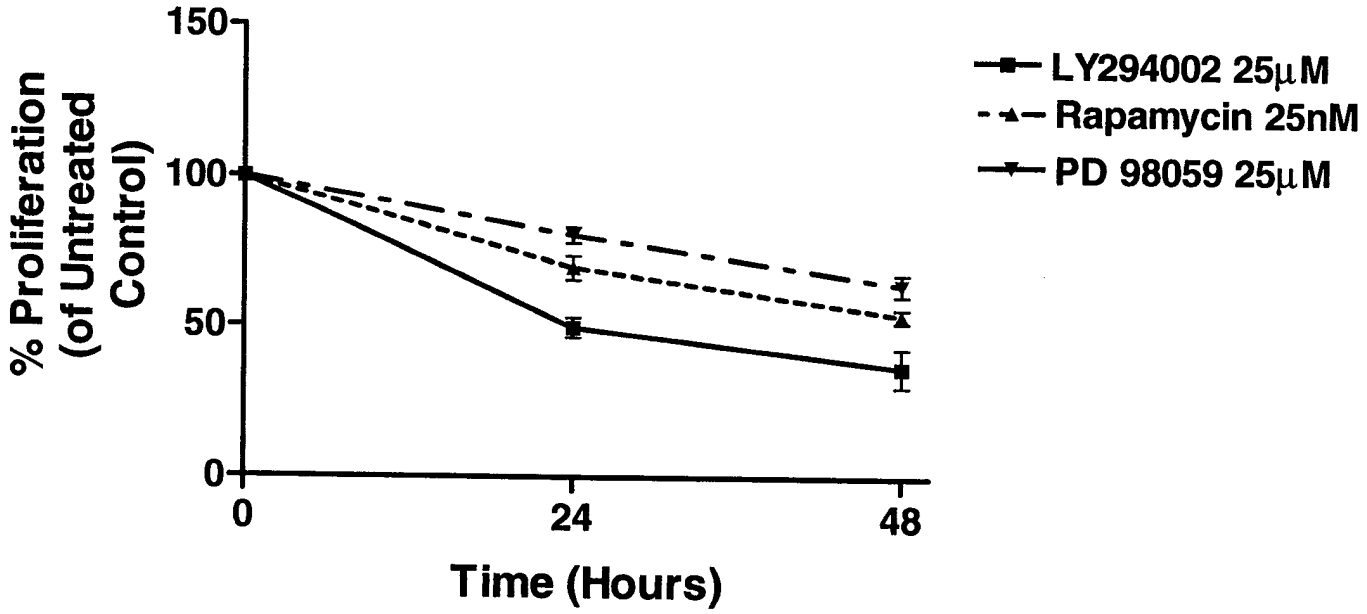
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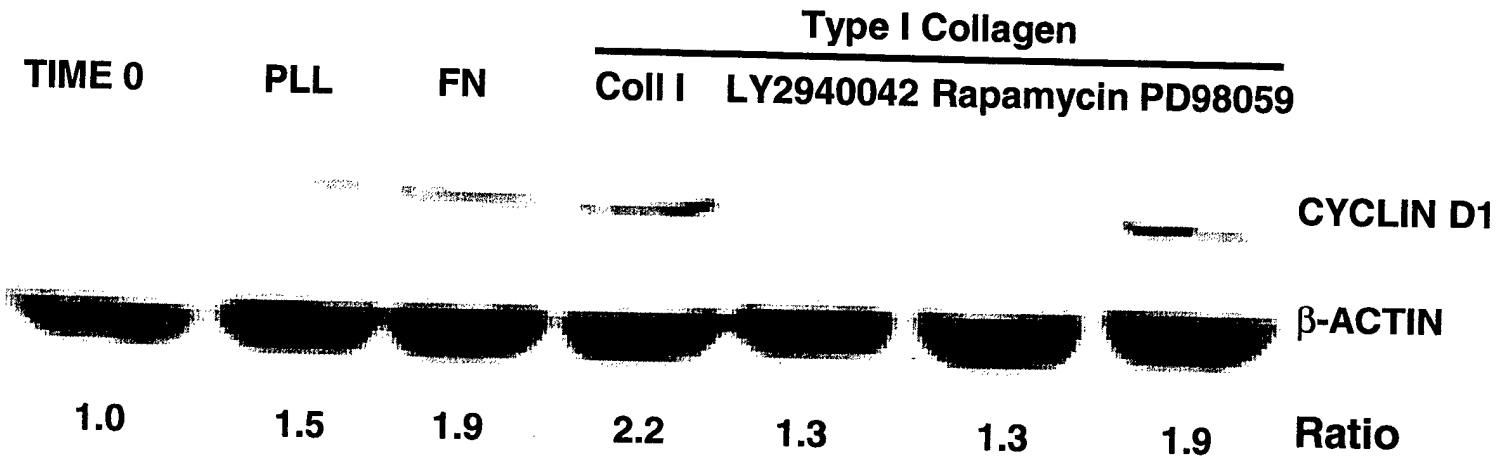
Figure 7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